HINDUSTAN ANTIBIOTICS

Bulletin

AUGUST 1961

VOL. 4

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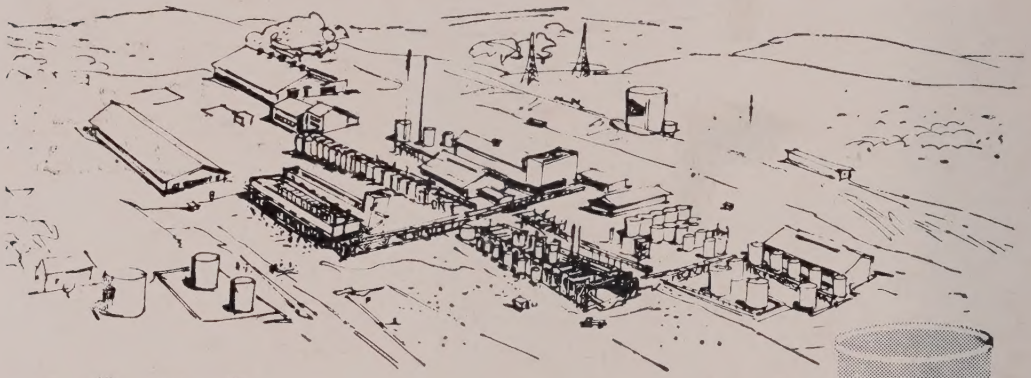
now offers from its Trombay plant



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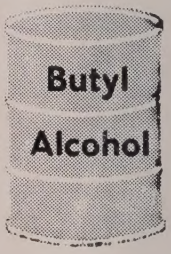

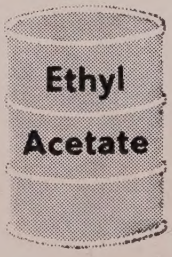

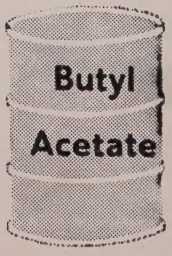

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HINDUSTAN ANTIBIOTICS

Bulletin

Vol. 4 **August 1961** **No. 1**

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EDITORIAL & ADVERTISEMENT OFFICES

Hindustan Antibiotics Ltd., Pimpri, Poona Dist.
INDIA

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The views expressed in this journal are those of the authors and do not necessarily represent those of the Company or of the editors.

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ADVERTISERS INDEX

CONTINUOUS FERMENTATION TECHNIQUES IN ANTIBIOTIC INDUSTRY

THE concept of continuous fermentation is not of recent origin since for several centuries the principles of continuous flow culture have been employed in the production of vinegar and yeast, and in sewage disposal. However, great interest has been aroused in continuous fermentation techniques with the advent of antibiotic fermentation industry. In recent years several international symposia have been held and reviews summarising the present state of our knowledge of continuous fermentation have been published.

The term continuous fermentation or flow culture denotes constant or regular addition of nutrients to the fermentor and simultaneous withdrawal of the product from the fermented broth. It may be a single stage homogeneous fermentation where the feed and product streams are equal and continuous. This is especially adapted to processes in which the product is directly associated with cell growth. In contrast, two stage and multiple stage processes involving recycling, etc., are also done. In the semi-continuous process, which is a modification of the single stage process, feeding and withdrawal are carried out intermittently. Malek and coworkers, Novick, Gerhardt, Maxon, Deindoerfer, Herbert and several others have contributed extensively to our knowledge on this subject.

Details about continuous flow culture for cell production such as food yeast, etc. and product formation from bacteria such as acetic acid, alcohols, and lactic acid, are well described, but there is relatively little published information on the production of antibiotics by continuous fermentation. There is little doubt, however, that much more valuable data have been obtained than what has been published. There are two methods adopted for the control of continuous culture : the turbidostat method and the chemostat method. The latter technique is the one principally used in most of the processes, because in this case the growth rate is regulated by limiting nutrient concentration. The optimum dilution rate is that which gives maximum productivity. As pointed by Malek and others, the physiology of the microorganism in continuous culture is markedly different from that in the usual batch cycle. Consequently, continuous flow culture has been used as an important tool in the study of mould metabolism.

Continuous fermentation in antibiotic industry is really an attractive proposition, since according to theoretical calculations, the production can be doubled as compared with the batch process. Some of the operations such as continuous sterilization and flow of media are already in use in the batch process, and hence switching on to continuous process to obtain the product also appeared attractive. Bartlett and Gerhardt studied production of chloramphenicol and penicillin by continuous fermentation. Pirt and Callow have published several papers on design of the apparatus for continuous flow culture for penicillin production and overcoming the difficulties encountered in the handling of filamentous fungi like *Penicillium chrysogenum*. They tried to show that the Monod-Novick-Szilard continuous flow culture principle developed for bacteria, may be applied to filamentous fungi also by proper regulation of the growth condition of the mould in the two stages. Brinberg and Grabovskaya worked on semi-continuous process for production of penicillin, strep-

tomycin and neomycin. Bhektereva and Kolsenikova reported a single stage method for continuous flow culture of *Streptomyces lavendulae*. Several other workers have published data on pilot plant work of this type.

The principal factor to be considered in most of the cases is that antibiotic production is not associated with growth. After the initial growth phase antibiotic production begins. The final product is synthesized in the cell and retained there, or excreted into the medium. A continuous one stage process to combine both in order to obtain single product has not been devised. This is one of the main reasons why the continuous single stage fermentations have not replaced batch process in antibiotic production. Brown, working with continuous production of streptomycin in a 1,000 hour fermentation, was able to obtain 70 to 80 per cent of that obtained in batch process. He was sceptical about the usefulness of this method in antibiotic industry. Another factor that may be discouraging in the continuous flow culture process is the origin of mutants which may overgrow the normal ones and become dominant. This is of lesser importance than the introduction of air-borne contaminants into the system in which case the process has to be started again. In spite of all these difficulties, the successes achieved in the production of industrial alcohol, food yeast, acetic acid, lactic acid, etc., by continuous flow culture are so striking that it has kept research workers looking for new techniques which would overcome the difficulties, and make continuous fermentation method for antibiotic production a success.

CORRIGENDUM

In the paper "Influence of some factors on penicillin titres in industrial fermentors : A statistical study" by R. S. Gondhalekar and R. S. Phadke, *Hindustan Antibiotics Bulletin* 3, 11 (1960), certain recomputed values are given below :

In Table II (p. 15) $r_{08} = -0.44688$

Table III. INTERFACTOR CORRELATION COEFFICIENTS

$r_{12} = +0.39408$	$r_{23} = +0.12271$	$r_{34} = -0.40502$	$r_{68} = +0.62113$
$r_{13} = +0.76758$	$r_{24} = -0.33544$	$r_{36} = -0.49497$	$r_{78} = -0.21359$
$r_{14} = -0.41752$	$r_{26} = -0.32494$	$r_{37} = +0.49468$	
$r_{16} = -0.61049$	$r_{27} = +0.64319$	$r_{38} = -0.54183$	
$r_{18} = -0.47475$	$r_{28} = +0.11866$	$r_{48} = +0.02343$	

On the basis of the recomputed values Equation (6) should read

$$Y_0 = 0.33X_1 + 3.47X_3 + 4.27X_7 - 21.66$$

Antimicrobial Substances from Flowering Plants

I. ANTIFUNGAL SUBSTANCES

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Introduction

THE problem of fungus and insect control in agriculture is of great antiquity. Nearly 3,000 years ago Homer spoke of "pest averting sulphur," and Democritus recommended sprinkling pure amura of olives on plants to control blight. These were probably the first suggestions of plant chemotherapy. The problem is, however, of great interest and importance even today for the loss in agricultural production due to plant diseases is considerable. In the United States alone the average annual losses on this account is estimated at $3 billion.[186] Diseases incited by such common parasitic fungi as the rusts, smuts, powdery mildew, root rot, foot rot or damping off, cause maximum harm to the plant.

Biological methods of plant protection would be ideal, but the use of plant extracts, antibiotics and other chemicals has been the more successful method in modern agricultural practice. The vascular plants offer vast scope for search for antimicrobial substances. The use of plant extract in the treatment of various human infections was well known in ancient system of medicine including the Indian and Chinese. Although most of the compounds so far isolated from higher plants have been found to be toxic to animals, by suitable modification of the structure or by synthesis of analogous compounds or by combination with less toxic compounds, therapeutically useful products may be developed. In the present series of reviews an attempt is made to summarize the status of our knowledge of those plant constituents which have antimicrobial and antiparasitic activity and thus may be useful in plant protection. Substances isolated from higher green plants that inhibit growth of pathogens at very low concentrations alone are discussed with particular emphasis on their chemistry and on studies made during the last ten years. Biochemical aspects and closely related synthetic work will be summarized.

Part I of the series is limited to compounds with antifungal activity; some crude antibacterial plant extracts reported prior to 1950 are, however, briefly discussed.

Crude Antimicrobial Extracts from Plants

The presence of substances with antimicrobial activity in higher plants has been reported by several early workers.[21,22,24,25,81,107,113] The discovery of the therapeutic value of penicillin and other antibiotics of microbial origin stimulated

a great deal of interest in the search for new compounds in the plant kingdom. Investigations have so far yielded over 800 antibiotic substances from micro-organisms and about 100 compounds with antimicrobial activity from higher plants. The first large scale screening of higher plants was carried out by Osborn.[120] Extracts from approximately 2,300 species of plants belonging to 166 families were tested against *S. aureus* and *E. coli* by the methods developed for the assay of penicillin.[1] Sixty three genera were found to contain substances which inhibited the growth of one or both organisms. Atkinson and Rainsford[11] recorded the results of a preliminary investigation of about 1,100 species of flowering plants native to Australia. Of these, fifty species showed activity against the gram positive *S. aureus*, but only four - *Drosera Whittakeri* and three species of *Persoonia* - were active against the gram negative *Salmonella typhosum* also. The activity was concentrated in the extracts of leaves and stems in the case of Drosera, while it was mainly in the extracts of berries of Persoonias. In the latter case, the activity was greater against gram negative than against gram positive bacteria. It was readily destroyed at pH 9 but persisted at pH 2 for several hours and was not destroyed by heating at 100° for at least 45 min. Crude extracts kept at 4° retained their activity for at least 8 months. Certain steam distillable oils from the flowers of *Chamaelaucium uncinatum* and from flowers, leaves and stems of *Darwinia citriodora* exhibited activity against *M. phlei*.[9] Essential oils of several species of flowering plants were also found inhibitory to *M. phlei* and *S. typhi*.[10] These workers did not test antifungal activity of the plant extracts.

Gilliver[65] tested the extracts from 1,915 flowering plant species (mostly the mixed extracts of leaves, stems, flowers and roots) for antifungal activity. Of the species tested 23 per cent in 113 families gave extracts inhibitory to the germination of the apple scab organism *Venturia inequalis*. Although species producing active extracts were found in great many of the flowering plants without relation to their taxonomic position, the more significant belonged to the following :-

Centrospermae (particularly Chenopodiaceae) ; Ranales (Ranunculaceae) ; Rhoeadales (Cruciferae) ; Rosales (Saxifragaceae, Hamamelidaceae, Pittosporaceae) ; Umbellales (Arabiaceae, Cornaceae, Umbelliferae) ; Primulales (Theophrastaceae, Myrsinaceae, Primulaceae) ; Tubiflorae (Solanaceae) ; Campanulales (Compositae) ; Liliflorae (Liliaceae, Dioscoreaceae). In some plants the compounds were generally distributed throughout ; in others they were localized *e.g.* in leaves. Some extracts killed the conidia. Drying in some cases resulted in loss of activity. Extracts of *Hedera helix* were particularly active. Other active plants included *Anemone nemorosa*, *Scrophularia nodosa*, *Caltha palustris*, *Primula vulgaris*, *Atriplex patula*, *Bellis perennis*, *Cornus sanguinea*, *fimpinella saxifraga*, *Scilla nutans*, *Allium cepa*, *Clinopodium vulgare*, *Castanea sativa*, *Ranunculus Ficaria*, *Salix purpurea*, *Tamus communis*, *Brassica* spp., *Chrysanthemum segetum*, *Medicago lupulina*, *Raphanus raphanistrum*, *Solanum dulcamara* and *Symphoricarpos albus*.

Sproston *et al.*,[144] tested extracts from 73 Vermont plants for antifungal and antibacterial activity. Virgin's bower (*Clematis virginiana*) corn, fireweed (*Epilobium angustifolium*), pigweed (*Chenopodium album*), nasturtium (*Tropaeolum majus*), musk melon (*Cucumis melo*), hardhack (*Spiraea tomentosa*), common chicory, touch-me-not (*Impatiens biflora*), tomato and common purslane, were amongst those examined for stimulatory or inhibitory activity to *S. aureus*, *E. coli*, *Sclerotinia fructicola*, *Colletotrichum lindemuthianum*, and *Rhodotorula glutinis*. *Impatiens*, *Cucumis* and *Tropaeolum* were mosti nhibitory against the fungi. The

majority of extracts were active against *Monilia* and *Rhodotorula*. Colletotrichum and bacteria were least affected.

Whereas the leaves and stems of banana (*Musa sapientum*) showed slight antibacterial activity, extracts of pulp and skin of ripe banana effectively inhibited the fungi *Fasarium oxysporum* and *F. lycopersici* and also *E. coli*, Staphylococcus, *Serratia marcescens*, *M. phlei*, *B. subtilis*, *Sarcina lutea*, *Rhodococcus roseus* and *Xanthomonas translucens*, and a low pressure distillable substance effectively inhibited *F. oxysporum*, *E. coli*, *S. lutea*, *M. phlei*, *B. cereus*, and *X. translucens*.[135] From sweet potatoes, three biologically active crude substances with both antifungal and antibacterial properties have been reported.[28] From the stems and roots of broad bean (*Vicia faba*) seedlings an acid stable antifungal substance was isolated in the crude form by Spencer.[142] Virtanen and Hietala[169, 170] screened many cultivated plants such as cereals, meadow grasses or winter turniprape, against *Sclerotinia trifoliorum* and *Fusarium nivale* in agar cultures. Oats, barley, wheat, winter turniprape, timothy, meadow fescue and maize all showed activity. The active principle in winter turniprape is probably a mustard oil.

Capek[31, 32] reported that the volatile oils and products of maceration from cinnamon, sweet marjoram, thyme, laurel, pimento, clove and coriander showed both bactericidal and fungicidal properties whereas anise and fennel oils were only fungicidal. Ginger and nutmeg were weakly active, while cumin, pepper and juniper oils were without any effect. The antimicrobial activity of anise and fennel volatile oils was due to anethole.

A large number of extracts from plants are reported to have food preservative properties.[87] The extract of avocado trees particularly from the roots of *Persea gratissima* or *americana* retarded the growth of food spoilage organism in cured meats, hams and luncheon meat. The extracts of iris bulbs, dry sage and canadian thistle enhanced the resistance of common pickling solutions to the development of pathogenic organisms. Plants of the genus *Krameria* such as *K. triandra* (Peruvian ratany) and *K. argentea* (Brazilian ratany) contain a heat stable nontoxic food preservative. The extracts of grape vine broad leaf gum (*Grindelia squarrosa*), pareira (*Chondrodendron tomentosum*), bitter-sweet (*Celastrus scandens*), Osage orange (*Maclura pomifera*), common agrimony (*Agrimonia grypose-pala*), white cedar (*Thuja occidentalis*), purple prairie clover (*Petalostemum purpureum*), spring avens (*Geum vernum*), St. John's wort (*Hypericum perforatum*), Iceland moss (*Cetraria islandica*), butternut tree (*Juglans cinerea*), galingale root (*Alpinia officinarum*) are reported to contain food preservative principles.

From over 300,000 species of higher plants known to occur in nature only about 2 per cent have been screened so far. Extracts of plants from 157 families have been reported to be active against microorganisms and about 20 per cent of these are active against fungi.

Several crystalline substances have been isolated from higher plants and the molecular constitutions established in many cases. It is evident that considerable chemical and biochemical work lies ahead as further screening reveals the presence of new compounds. Mention may be made here of the excellent reviews by Nickell[118] and Cavallito.[35] Erdtman[51] has reviewed the chemistry of heartwood constituents of conifers. A good monograph on Indian medicinal plants is that by Chopra.[42] Some general conclusions from the voluminous literature of this field published in the last eighteen years

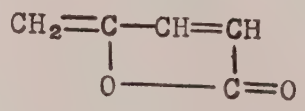
are : (*a*) There is no relationship between the antimicrobial activity and the taxonomic position of the plant ; (*b*) air drying generally reduces or even destroys the activity ; (*c*) seasonal variations affect the activity ; (*d*) the activity may be affected by extraction procedure ; and (*e*) many of the antimicrobial substances such as isothiocyanates and benzoxazolinones occur as the glucosidic precursors and may be liberated on damage to the plant tissues.

Antifungal Substances

Typical antifungal compounds isolated from plants are discussed below. Investigations prior to 1950 are only briefly treated, and the chemistry of compounds reported in the last decade is dealt with in greater detail.

LACTONES

The lactone, protoanemonin (5-methylene-2-oxodihydrofuran) (I) occurs in the extracts of butter cup and *Anemone pulsatilla*.[12, 136] In the pure state it readily polymerizes to the dimer anemonin (II) and other higher polymers. The structures of these compounds have been established,[8] and their syntheses reported by several workers.[89, 114, 137] These compounds are active against both gram positive and gram negative bacteria, fungi, yeast and protozoa at very low dilution,[12, 21, 23, 75, 131] the fungistatic concentration of protoanemonin against *Candida albicans* being 0.008-0.016 mg./ml. The protoanemonin activity has been observed in many Ranunculaceae such as *Anemone nemorosa*, *Ranunculus bulbosus* and *R. acris*.

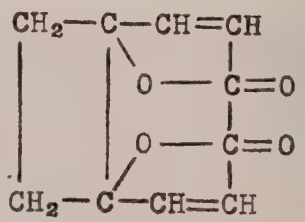


I

Parasorbic acid (III), a dextrorotatory unsaturated lactone (δ-hexenolactone) was isolated from berries of mountain ash, *Sorbus aucuparia*.[46] Kuhn and Jerchel[99] established its structure which was confirmed further by synthesis.[71, 111] The D1-form of the lactone completely inhibits the growth of species of *Fusarium*, *Phycomyces* and *Nematospora* at a concentration of 1 mg./ml. and .markedly at 0.1 mg./ml.[29]

Plumericin, C_{15} H_{14} O_6 isolated from the roots of *Plumeria multiflora* is active against several species of fungi and gram positive and gram negative bacteria. The compound is a neutral, unsaturated lactone containing four masked acid groups and three unsaturated bonds per molecule.[104]

The unsaturated lactones probably comprise of one of the largest groups of natu-



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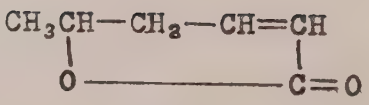


III

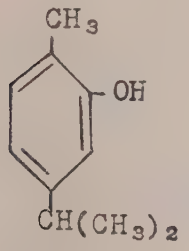
rally occurring compounds exhibiting diverse types of biological action. This structural feature is found in heart aglycones, fish poisons of the furocoumarin type, antibiotics of microbial origin such as penicillic acid[20], and patulin.[187] The unsaturated lactone group appears to be essential for the antibiotic action of these compounds since the hydrolysis of the lactone or reduction of its double bond destroys or considerably reduces the activity. Since the lactones are inactivated by cysteine and related substances, their antibiotic properties are probably due to their ability to interfere with the normal function of the sulphydral groups in microorganisms.[36, 38]

QUINONES

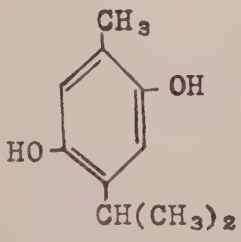
The occurrence of the phenol, carvacrol (2-hydroxycymene) (IV), and the quinones, hydrothymoquinone (2, 5-dihydroxy-*p*-cymene) (V) and thymoquinone (2-methyl-5-*iso*propyl-1:4-benzoquinone) (VI) has been reported in the heartwood. The compounds were found toxic to wood decaying fungi and to the germination of conidia of common root rot fungus and to the blue stain fungi.[54]

Relatively few benzoquinones have been found in higher plants.

Among the naphthoquinones, plumbagin (2-methyl-5-hydroxy-1, 4-naphthoquinone) (VII) has been isolated from the roots of *Plumbago europaea* L.,[48] *P. zeylanica*, and *P. rosea* L. Fieser and Dunn[57] synthesized it. The quinone inhibits *S. aureus* and *S. pyogenes* in a concentration of 0.01 mg./ml. in liquid media,[129] and a number of human pathogenic fungi such as *Coccidioides immitis*, *Histoplasma capsulatum*, *Ctenomyces radians*, *Trichophyton ferrugineum* in a concentration of 0.025 mg./ml.[128] The closely related 2-methoxy-1:4-napthoquinone (VIII) occurring in the garden balsam (*Impatiens balsamina* L.) also shows strong antifungal



IV



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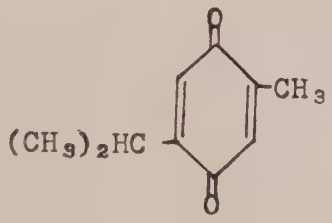

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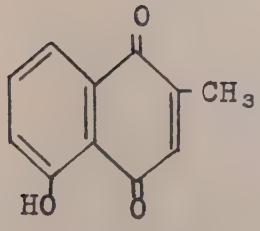

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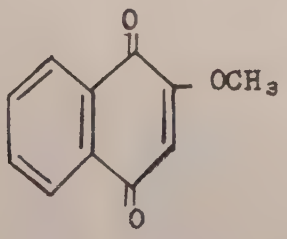
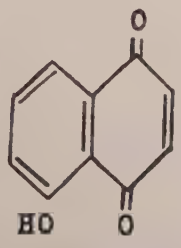


VIII



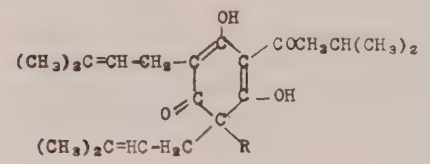
IX

properties against several fungi.[105] The microbial antibiotics fumigatin,[4] javanicin,[7] and spinulosin[5] are methoxyquinones and belong to this group. Other methoxyquinones having marked antifungal activities have also been reported[43, 64, 121].

Juglone, 5-hydroxy-1, 4-naphthoquinone (IX), isolated from *Juglans nigra* L., *J. cinerea* L., and *J. regia* L., has been reported effective against ringworm infection incited by *Microsporum audouini*, *Trichophyton* spp. [66, 184].

KETONES

Humulon (X) and lupulon (XI), the unsaturated cyclic ketones, are the preservative principles of hops (*Humulus lupulus* L., Moraceae).[70, 80, 180, 181] These compounds were reported active against twelve species of fungi, but complete inhibition of mycelial growth was, however, difficult to achieve. The large scale use of hops in beer production suggests these compounds are non-toxic. Their low solubility in water may make them desirable for topical applications where a more soluble substances would be dissolved too rapidly. Lupulon is colourless, odourless and tasteless. These properties favour its possible medicinal use in foods.[112]



X, R = OH. XI, R = $-CH_2-CH=C(CH_3)_2$

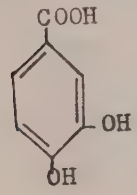
PHENOLIC COMPOUNDS

The resistance of plants to fungal attack markedly affects the yield and partly the quality of the crops.[173] In 1905, Biffen[19] suggested that resistance to yellow rust, *Puccinia glumarum* in wheat was inherited as a Mendelian character. It is now generally recognized that many cases of plant resistance are gene controlled. Considerable knowledge has been gained on the mode of inheritance and nature of resistance.[178] Greater attention is now being given to biochemical or physiological differences to discover the basis of resistance of plants to diseases since the histological differences between varieties were inadequate to account for the variation in resistance characteristics. In the early stages of plant growth antifungal substances are found to be present in sufficient concentrations in all plants investigated. Knowledge of the relation between the presence and nature of the antifungal factors and development of resistance to pathogenic fungi is therefore of importance in plant breeding for evolving genetic lines of resistant varieties, and also in studies on plant metabolism.

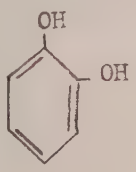
The penetration of the host by the fungus is the first step in host-parasite interaction which may lead to establishment of infection and subsequent disease development. Cook[44] in 1911 suggested that at the site of penetration the activity of polyphenol oxidase may release phenols into the substrate which inhibit progress of pathogens. The wheat variety Khapli resistant to stem rust (*P. graminis* Pers.) was reported to have higher phenol content than the susceptible varieties.[116, 117] The high resistance of onion varieties (*Allium cepa* Linn.), in which bulbs and scales are pigmented red or yellow, against onion smudge caused by *C. circinans* has been shown to be due to the presence of protocatechuic acid (XII) and catechol[102, 103] (XIII). These substances being water soluble and toxic, diffuse to the site of infection on the surface of the host and prevent germination and penetration. The cell sap of wheat varieties *Triticum timopheevi* and *T. monococcum* immune or highly resistant to brown rust (*Puccinia triticina*) contain protocatechuic phenols while the susceptible varieties are poor or completely devoid of these compounds.[88] The presence of a toxic substance in the extract

of host tissues, however, may not necessarily mean enhanced resistance of the host to a pathogen. The toxic material may be present in a non-lethal form such as glycoside, or it may be destroyed in advance by excretion of the invading organism or may be present in some other portion of the cell not attacked by the pathogens.[178]

Chlorogenic acid (XIV) and caffeic acid (XV) have been reported to be associated with the immunity of white potatoes from attack by Race 1 of *Helminthosporium carbonum*. Potato tuber tissues inoculated with the fungus produced 2 or 3 times as much chlorogenic acid and caffeic acids as did noninoculated control tissues.[97, 98]



XII



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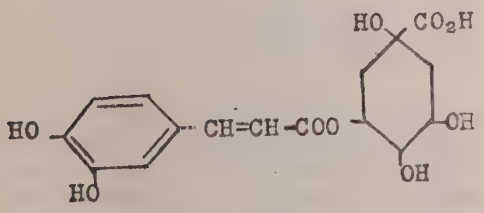
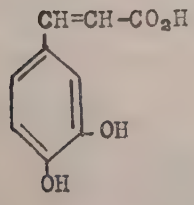


XIV



XV

The accumulation of chlorogenic acid, caffeic acid, methyl caffeate, umbelliferone, scopoletin, ascorbic acid and ipomearone in healthy sweet potato tissue adjacent to that spoiled by *Ceratostomella fimbriata* have been reported.[153-156] These substances are considered to be responsible for the resistance of sweet potatoes to this fungus. The respiratory enzymes appear to be inactivated during the oxidation of sweet potato polyphenols. Phenolic compounds are also produced by the plants in response to inoculation with the organism. Caffeic or chlorogenic acid or both are also present in potato tuber extract found to inhibit *Citrum roseum* and *Myrothecium verrucaria*.[96] The press juice of potato leaves (Aquila variety) is found to inhibit late blight (*Phytophthora infestans*). This inhibiting effect is mainly due to chlorogenic acid and accounts for the resistance of this variety to late blight.[173]

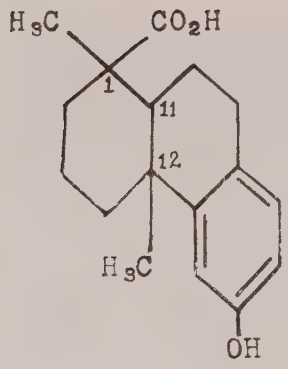
The high durability of the wood of the tree *Dacrydium cupressinum* (Rimu) is considered due to the presence of the fungicidal phenol podocarpic acid (XVI). Ferruginol (XVIa) is another fungicidal phenol found in the heartwood of *Podocarpus ferrugineus* in New Zealand. The configuration at C_{11} and C_{12} in both these compounds in *trans*.[52]

The wood of *Podocarpus totara* containing a diterpene alcohol, totarol ($C_{20}H_{30}O$), is highly resistant to wood decaying fungi and marine borers.[52]

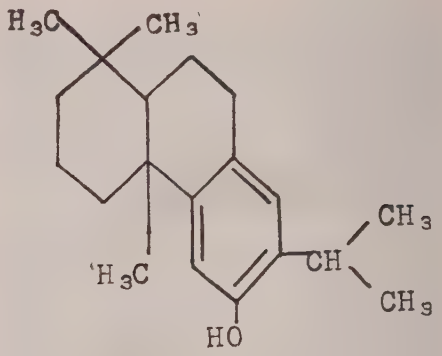
ACIDS

Cyclic acids:—From the heartwood of conifers Erdtman and others,[34, 49] isolated two strongly antifungal isomeric acids, chamic (XVII) and chaminic (XVIII) acids.

Amino acids:—Canavanine (XIX) an amino acid isolated from Jack beans, has



XVI

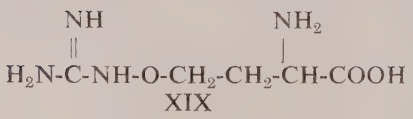


XVIa

been reported active against most strains of *Neurospora* at about 6 p.p.m.[79a]

XVII

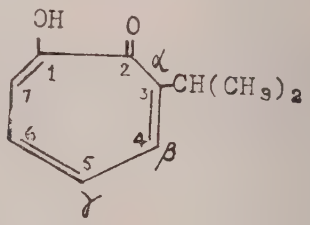
XVIII



XIX

TROPOLONES

Sowder[141] found the water extracts from Western red cedar highly toxic to the wood decaying fungus *Lentinus lepideus*. A crystalline compound isolated from the red cedar was reported inhibitory to *Fomes annosus*, a wood decaying fungus, at concentrations of 0.05 to 0.07 mg./ml[6]. Studies by Erdtman and others have led to the isolation of three highly active antifungal simple tropolone derivatives from the heartwood of *Thuja plicata*, the North American Western red cedar. These are ∝ (3-*iso*propyl) (XX),[67] β-(4-*iso*propyl) (XXI)[3, 119] and γ-(5-*iso*-propyl) (XXII)[53] thujaplicins. Fungicidal



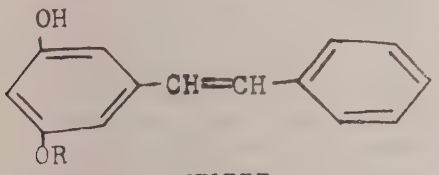
XX, α-
XXI, β-
XXII, γ-

studies indicated that γ-thujaplicin was thousand times more active against decay fungi than phenol. The β-form was most active and all were more active than sodium pentachlorophenol.[127] Other species of the genus Thuja such as *T. occidentalis*, *T. standishii*, and *T. dolobrata*, were also found to contain one or more of these compounds.[49] The highly resistant nature of these woods to decay has been attributed to the presence of thujaplicins.

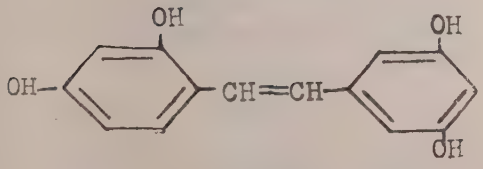
STILBENES

Pinosylvine (*trans* 3-5-dihydroxystilbene) (XXIII) and its monomethylether (XXIV) occur in the heartwood of coniferous trees *Pinus silvestris* constituting

together about 0.8 per cent of the material.[50] They are inhibitory to the growth of *Polyporus vaporarius*, *Coniophora cerebella* and *Lentinus squamosus* at 0.05 to 0.2 mg./ml. on malt agar.[55] The two compounds also exhibit bactericidal properties against *S. aureus* and *B. subtilis* at 0.05 mg./ml. and to *S. typhimurium* in about 0.5 mg./ml. The dimethyl ether is devoid of antibacterial activity. The M.L.D. of pinosylvine is 1-2 mg. injected intraperitoneally into a 15 g. mouse as compared to 2.5 mg. for phenol.[63] The greater resistance to fungal attack of the heartwood than the sap wood of Scotch pine is due to the presence of these substances ; in cases of severe attacks their concentrations are much lower. The inhibitory activity of sawdust from a variety of coniferous trees against the cellulose decomposing fungi *Sporocytophaga myxococcoides* is probably due to pinosylvine and its monomethyl ether.[84] The toxicity of hot water extracts from 11 species of tropical woods of Central and South America and three north American woods to



XXIII, R = H
XXIV, R = CH_3



XXV

Lenzites trabea and *Poria microspora*, has been rated as follows :- *Clarisia racemosa*, very toxic ; *Chaetoptelea mexicana* and *Quercus copeyensis*, toxic ; *Persea pallida*, *Terminalia amazonia*, *Nectandra rectinervia*, *Ocotea tonduzii*, *Brosimum utile*, intermediate ; *Carapa guianensis*, *Rhizophora mangle* and *Humiria* species, non toxic.[182] Hot water extracts of the north American woods Douglas fir, Black locust and white oak indicated decreasing toxicity in the order listed. However, no correlation could be established between the durability of the woods as indicated by the *in vitro* tests and the actual service data. The antifungal agents in these woods may possibly be pinosylvine or related compounds.

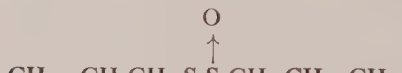
The wood of the Osage orange, *Toxylon pomiferum* is reported to contain about 1 per cent of 2,3′,4,5′-tetrahydroxystilbene (XXV). The substance inhibits completely the growth of *Myrothecium verrucaria*, *Pullularia pullulans*, *Microsporum canis* and *Trichophyton mentagrophytes* at a concentration of 0.2 mg./ml. The great durability and resistance to decay of this wood is believed to be due to the presence of the tetrahydroxy compounds.[13]

SULPHOXIDES AND THIOSULPHINATES

The common garlic, *Allium sativum* has been atttributed great therapeutic virtues in ancient systems of medicine as well as in modern scientific literature. Fortunatov[60] claimed garlic extracts better than penicillin for the treatment of some throat infections. The earlier voluminous literature on the antimicrobial activity of garlic is rather indefinite and most of the claims have been poorly substantiated.

The antimicrobial activity of garlic extracts has been claimed to be due to the presence of diallyl sulfide,[152] unstable sulfur in alkylpolysulphides,[90] bacteriophage,[85] unsaturated aldehydes,[33, 174] or to "phytoncides".[151] The high antibacterial activity of garlic in cylinder plate assay was observed by Cavallito and Bailey[37] in 1944. They isolated the antimicrobial factor, allicin, as a colourless liquid and established its structure[39] as allyl-2-propene-1-thiol-sulphinate (XXVI)). The compound was active against gram positive and gram

negative organisms and was particularly inhibitory to fungi, killing *Trichophyton gypseum* and *Microsporum audouini* at a concentration of 0.001 mg./ml. Antifungal studies on synthetic preparations such as methyl, ethyl, *n*-propyl, *i*-propyl, *t*-butylethyl, *n*-butyl, *n*-amyl and allyl obtained by the controlled oxidation of the corresponding disulphides indicated that the lower molecular weight thiolsulphinates (2, 4, and 6 carbon atoms) were almost equally effective in inhibiting gram positive or gram negative bacteria but as



XXVI

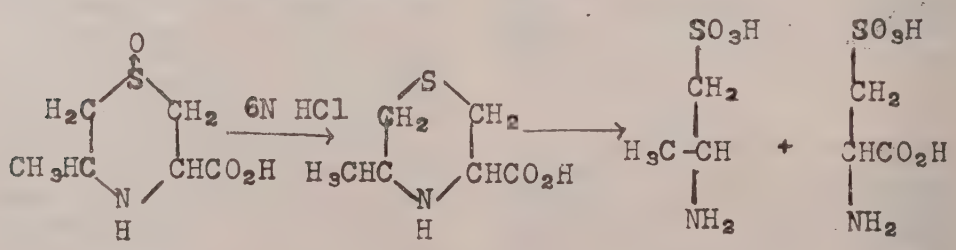
the chain length increased, activity against gram negative organism decreased while that against gram positive bacteria increased. Branching resulted in lowering of activity. The order of activity was found to be *n*-propyl > *iso*-propyl > *t*-butylethyl derivatives.[138] The thiolsulphinates appear to exert their antibiotic effect through binding of essential sulfhydryl groups.[138] Toxicity tests in mice for allicin in aqueous solutions gave LD_{50} of 60 mg./kg. intravenously and 120 mg./kg. by subcutaneous administration.[37]

Rao and coworkers[124] observed garlic extracts to be inhibitory to *M. tuberculosis* (human strain B 52, H_1, Kasauli). The activity was comparatively stable in the presence of blood and artificial gastric juice but was inactivated by artificial pancreatic juice and it inhibited the milk clotting activity of papain and the amylolytic activity of *B. amylase* probably by attacking the -SH groups of enzymes.[125] Alkaline phosphatase,[45] invertase,[45] urease,[150] succinic dehydrogenase[150] and blood peroxidases[157] are inhibited by garlic extracts, but carboxylase and cytochrome oxidase activity is not affected.[150] Allicin also inhibits urease.[2] Invertase activity of red clover extract, on the other hand, is increased by garlic extracts.[113] Enzyme inhibition studies on twentyeight enzymes in

XXVII

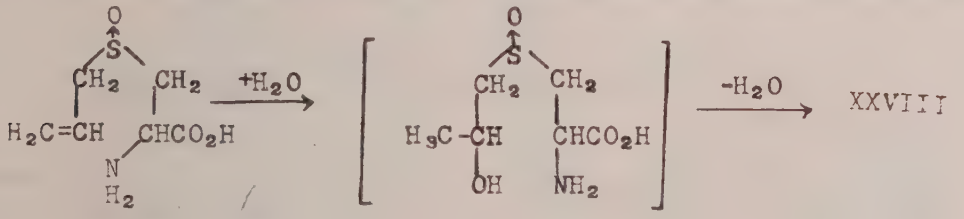
the presence of various concentrations of alliin (S-allyl-L-cysteine sulphoxide) (XXVII) and allicin (XXVI) have shown that all enzyme inhibitory effects observed with garlic extracts could be explained by the presence of allicin in them.[188]

Allicin occurs in plant in the form of the stable precursor alliin which gets cleaved into allicin under the influence of an enzyme, alliinase, when the plant in crushed.[40, 145] Two molecules of alliin yield one mole of thiosulphinate and two each of pyruvic acid and ammonia.[146] Both natural alliin which is identical with the synthetic (+)-S-allyl-L-cysteine sulphoxide and its three optically active isomers have been synthesized.[147, 148] The specific constituents of garlic have been reviewed by Stoll and Seebeck.[149]



XXVIII XXIX XXX XXXI

In 1956 Virtanen and Matikkala[168] isolated from ethanolic extract of onion, *Allium cepa*, a sulphur containing amino acid $C_6H_{11}O_2NS$ (XXIX) as its hydrochloride after hydrolysis with 6*N* HCl. The compound could be oxidized to the corresponding sulphoxide $C_6H_{11}O_3NS$. HCl (XXVIII) by hydrogen peroxide and the sulphoxide reduced with Raney nickel back to XXIX indicating that an amino acid sulphoxide and its thioether were involved. The sulphoxide was also found as an original amino acid in onions.[110]

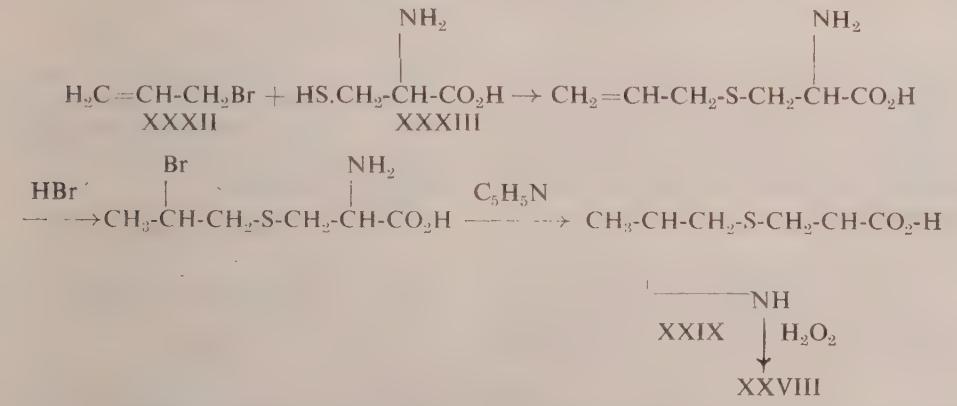


XXVII

It yielded 2-aminopropane-1-sulphonic acid (2-methyltaurine) (XXX) and cysteic acid (XXXI) on hydrolysis with hydrochloric acid. These results suggested 3-methyl-1, 4-thiazane-5-carboxylic acid 1-oxide structure for the sulphoxide (XXVIII), and 3-methyl-1,4-thiazane-5-carboxylic acid (XXIX) for the thioether.[158, 165, 166]

Since XXVIII had the same composition as alliin it was termed cycloalliin. Biosynthetically it could be formed from alliin by the simultaneous addition to and elimination of a molecule of water from alliin (XXVII).

Cycloalliin has been synthesized[166] from allyl bromide (XXXII) and cysteine (XXXIII).

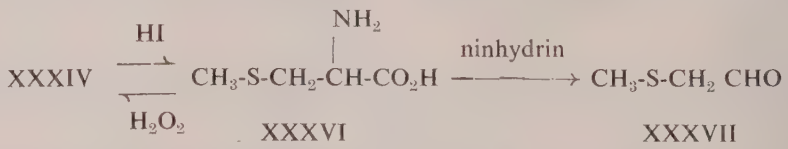


Cycloalliin represents a new class of natural amino acid found in all parts of Allium species investigated. Onions contain from 1.4-3.2 g. of cycloalliin hydrochloride per kg. [166] The antibiotic activity of onions is slightly weaker than that of allyl thiolsulphinate (XXVI) formed from S- allyl cysteine sulphoxide (XXVII) present in garlic.[39] Fifty to hundred mg. of freshly crushed onion in 1 ml. of nutrient solution completely inhibits the growth of *S. aureus*.[167] As in case of garlic if the enzymes in the whole onion

are inactivated before crushing, the antibiotic activity is very poor. Recently Virtanen and Matikkala isolated two new sulphoxides from chilled onions by extraction with cold methanol and chromato-

XXXIV

graphy of the ether extract over ion exchange columns and established their structures[167] as S-methylcysteine sulphoxide



XXXIV XXXVI XXXVII

(XXXIV) (MCSO) and S-*n*-propylcysteine-sulphoxide (XXXV) (PCSO) (dihydroalliin).

The structure of MCSO was confirmed through its reduction to S-methylcysteine (XXXVI) and oxidation of XXXVI with ninhydrin to S-methylthioacetaldehyde (XXXVII) according to the procedure of Virtanen.[172]

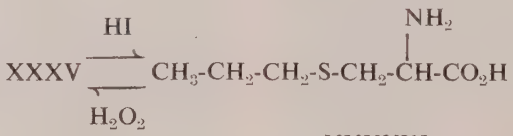
Reduction of XXXV gave thioether which on paper chromatographic comparison was identical with synthetic *n*-propylcysteine (XXXVIII).[167]

The sulphoxides are the parent compounds which are readily converted to the corresponding thiolsulphinates under the influence of enzymes liberated on crushing the onion and these are responsible for the strong antimicrobial action.[158] Antitubercular activity has also been reported for extract of onions.[69] One g. of chilled peeled onion contains about 50-60 μg. of PCSO and 200 μg. of MCSO.

Amongst the naturally occurring alkyl cysteine sulphoxides methyl cysteine sul-

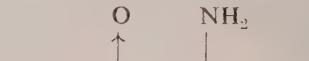
XXXV

phoxide occurs most widely. It occurs in several members of the Liliaceae and Cruciferae families and occasionally in Compositae (in *Lactuca sativa* L.), Umbelliferae (in *Cryptotaenia japonica* Hasskarc) and Leguminosae (*Phaseolus vulgaris* L.)



XXXVIII

Propyl - and allyl - cysteine sulphoxides have been found only in Liliaceae so far. Ethyl cysteine sulphoxide (XXXIX) is reported only in *Ipheon uniformum*, a member of Liliaceae.



XXXIX

A partial purification of an enzyme for degradation of alkyl cysteine sulphoxide from onions to pyruvic acids (as in the case of garlic by the enzyme alliinase) has recently been carried out.[101] The enzyme appears to require pyridoxal phosphate as a cofactor but shows no stimulation of activity by various metals. Carbonyl group reagents such as hydroxy-

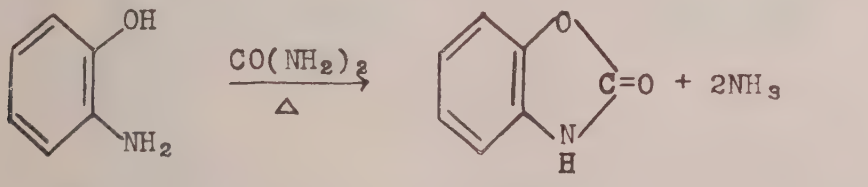
lamine and hydrazine inhibit the enzyme. The sulphydryl group inhibitors show almost no inhibition. The purified enzyme gives a single protein peak at two different pH in electrophoresis.

BENZOXAZOLINONES

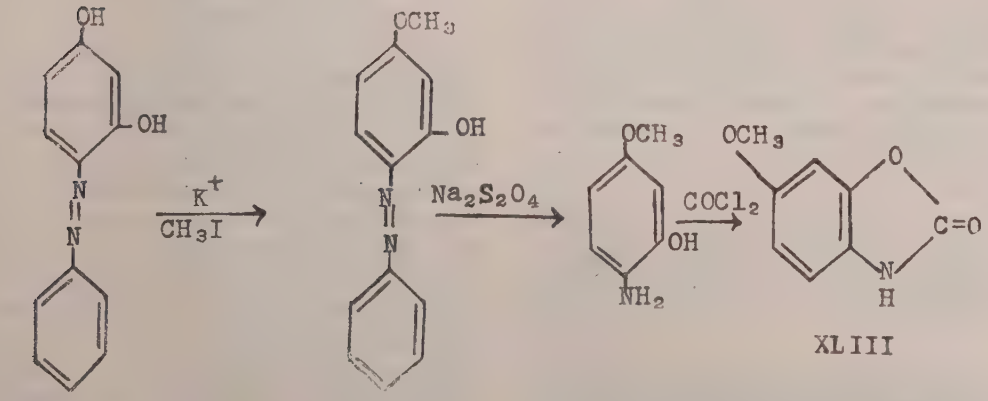
Fusarium nivale, also called snow mould, grows as a white or reddish grey mould on rye or wheat under snow in northern Europe frequently causing rot of a large group of plants. It also causes the serious patch disease of turf in lawn and golf courses. In countries with heavy snow fall *F. nivale* is particularly destructive to autumn snow cereals on which it causes the disease called snow mould, and large areas of grasses and cereals are destroyed. The fungus affects the overwintering of rye in snow covered fields and possibly has greater effect on the winter hardness of winter cereals than frost.[159]

From 5-6 days old germinated rye seedlings, an antifusarium factor was isolated by extraction of the homogenized seedlings with ether in the presence of acid.[159] On the basis of its empirical formula, chemical and physical characteristics (e.g. aromatic ring, negative ninhydrin test, absence of carboxyl group and ultraviolet absorption peak at 270 mμ) it was considered to be 2(3)-benzoxazolinone (BOA) (XLI). The substance was synthesized by fusing *o*-aminophenol (XL) and urea according to the procedure of Bywater.[30]

The compound inhibited the growth of *F. nivale* completely at 0.5 mg./ml. and weakly at 0.1 mg./ml. The benzoxazolinone content of rye seedlings is about 0.01 per cent and its isolation probably represented the first example of a benzoxazolinone found in nature. Based on its strong absorption at 275 mμ, Hietala



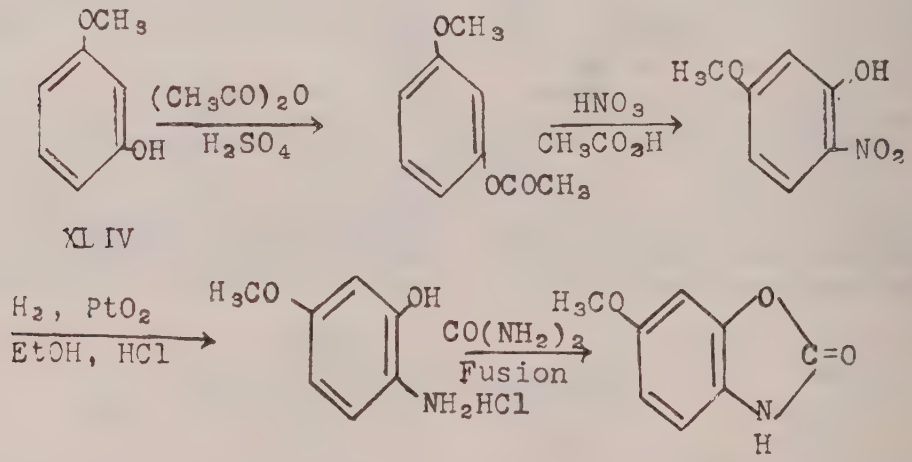
XL XLI



XLII XLIII

and Virtanen[72] developed a spectrophotometric procedure for the estimation of BOA. Another antifungal compound, 6-methoxy-2 (3)-benzoxazolinone (XLIII) (MBOA) has been isolated from maize and wheat plant[171] and from corn plant,[106, 139] and it has been synthesized[74] from *p*-benzene-azo-resorcinol (XLII). An alternative synthesis from resorcinol monomethyl ether (XLIV) has also been reported.[140]

in boiling water, and purified by countercurrent extraction with butyl alcohol-water solvent system. Enzymatic hydrolysis of XLV gave the aglucone $C_8H_7O_4N$ (XLVII) while alkaline fusion and strong acid hydrolysis yielded $O\text{-}NH_2C_6H_4OH$.[160] The glucosidic precursor of 6-methoxybenzoxazolinone, $C_{15}H_{19}O_{10}N$ (XLVI) has also been isolated from maize seedlings and wheat plants. Press juice from maize seedlings on ether extraction gave the



The amount of the material present in plants is related to the age of the plant, the tissue from which it is extracted and to the strain of the corn used as source. Chromatographic purification of the ether extract over aluminium oxide and measurement of the molar absorption at 285 mμ has been used for the quantitative estimation of the resistance factor 6-methoxybenzoxazolinone.[17]

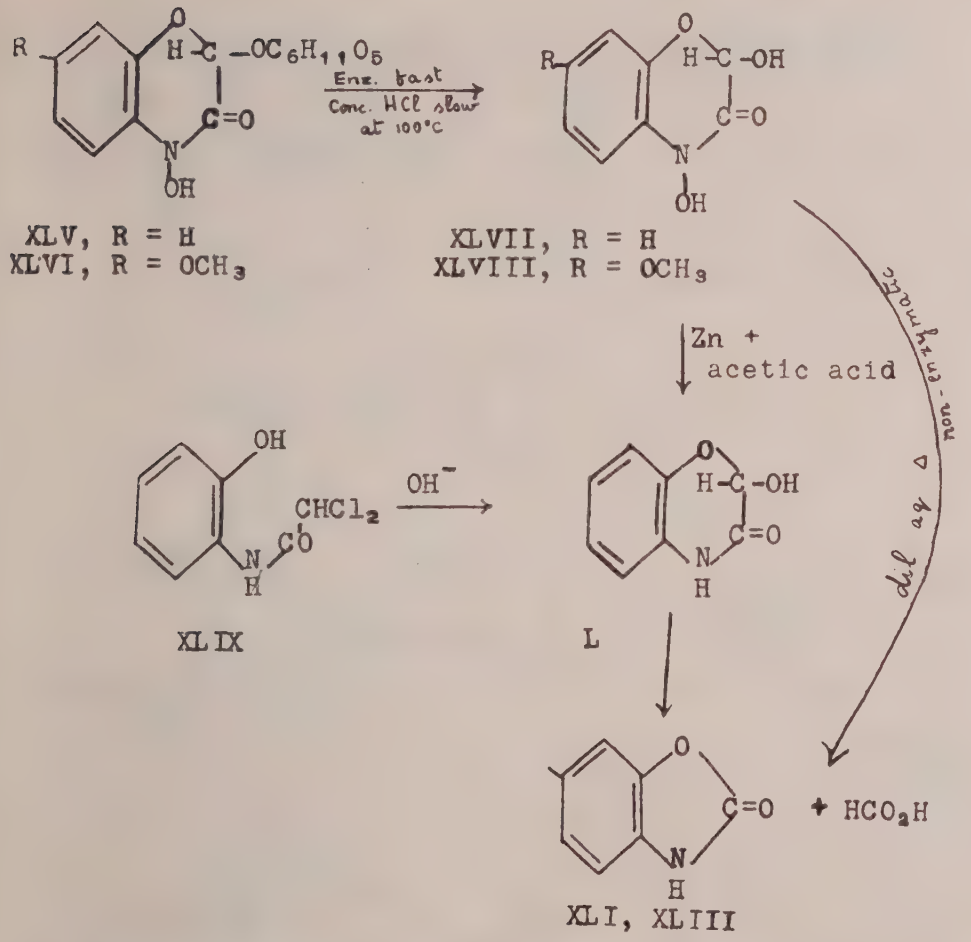
MBOA inhibits the growth of a variety of fungi, bacteria and insects in addition to the European corn borer larvae *Pyrausta nubilalis* (Hbn).[14-16]

The glucoside precursor of BOA, $C_{14}H_{17}O_9N$ (XLV) was isolated from rye seedlings after destruction of the enzyme aglucone, $C_9H_9O_5N$ (XLVIII) needles, m.p. 156-57°. The identity of the glucoside precursors of MBOA is maize and wheat seedlings was shown by two dimensional paper chromatography.[175]

Benzoxazolinone (XLI) from rye seedlings and 6-methoxybenzoxazolinone (XLIII) from wheat and corn plants[176] are formed from glucosidic precursors (XLV) and (XLVI) respectively through enzymatic and chemical reactions to form aglucones (XLVII) and (XLVIII).[73, 76, 77, 161]

The evidence for the structure of glucosides and aglucones are :

(a) The sugar was established as glucose by paper chromatography.



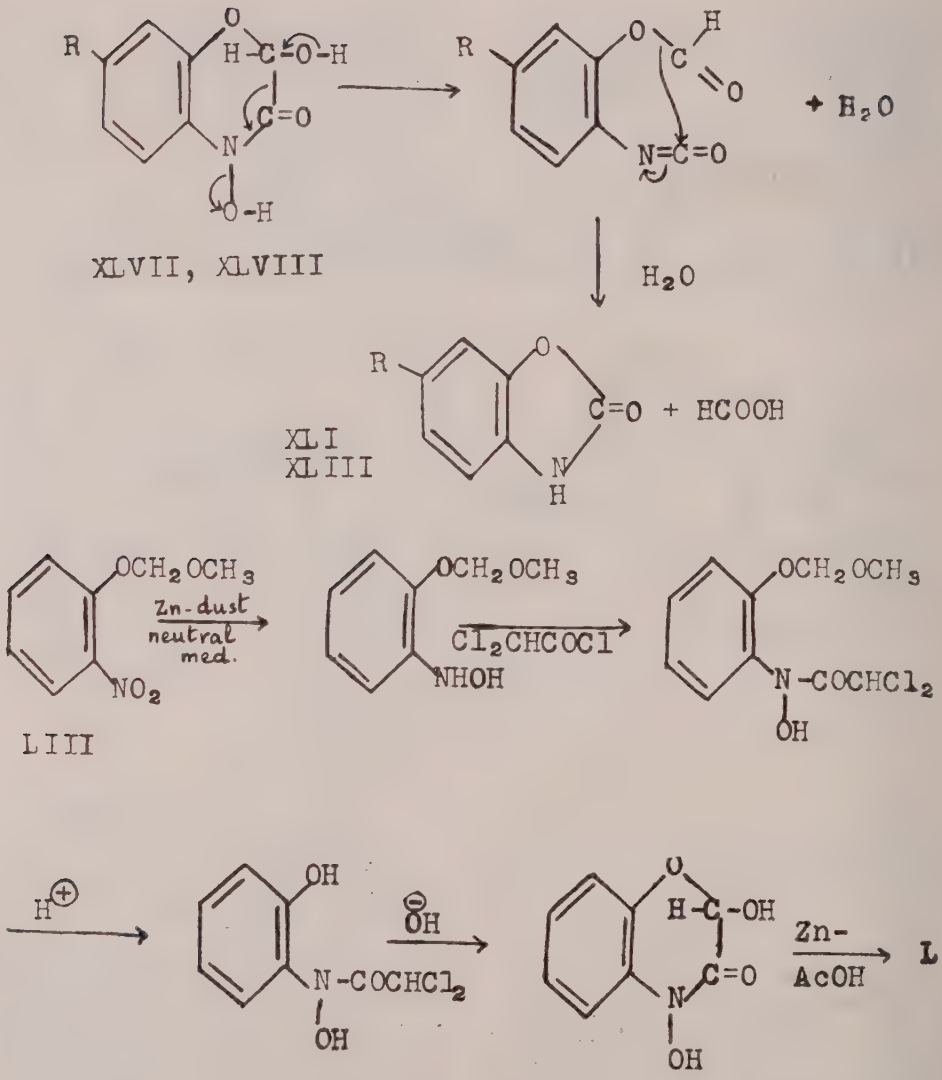
(b) The aglucone reduction product (L) was synthesized by heating *o*-(dichloroacetamido)-phenol (XLIX) in dilute sodium bicarbonate solution. This confirmed the structure of aglucone formed enzymatically from the glucoside.

(c) Both aglucone and glucoside gave strong violet colour with ferric chloride. The aglucone reduction product (L) gave no such colour and BOA was not formed from it upon heating its water solution. The NOH group had apparently been reduced to an NH group.

The aglucone had an antifusarium effect about one half of BOA. The antifusarium effect of glucoside was very weak. Although no evidence exists, a possible mechanism for the formation of BOA and MBOA from their respective aglucones may be suggested as in page 18.

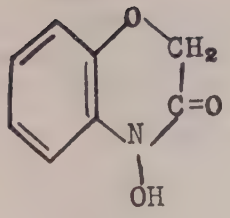
The structure of the aglucone (XLVII) has been confirmed by synthesis, starting from *o*-(methoxymethoxy)-nitrobenzene (LIII).[77]

In an effort to obtain more effective antifusarium compounds, synthesis of several

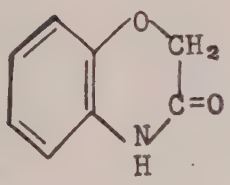


1, 4-benzoxazine derivatives were undertaken by Honkanen and Virtanen,[78] and their growth inhibiting effect on *Fusarium nivale* investigated.

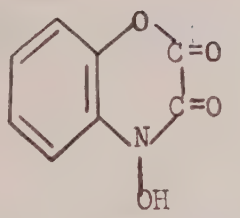
The compound (LVIII) containing the completely hydrogenated heterocyclic ring was most effective. A comparison of the compounds (LIV) with (LV), and (LVI) with (LVII)indicated that the compounds with NOH group are about twice as effective as the corresponding compounds with the NH group. The NOH compound corresponding to LVIII should, therefore, be most effective on this basis. This compound has not been synthesized so far. The oxidation of 2 and 3 carbon atoms reduced the activity. The compound (LIV) inhibited the growth of *S. aureus*, *P. fluorescens* and *E. coli* in a concentration of 1 mg./ml.



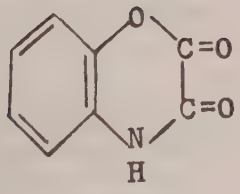
LIV



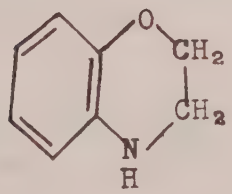
LV



LVI



LVII



LVIII

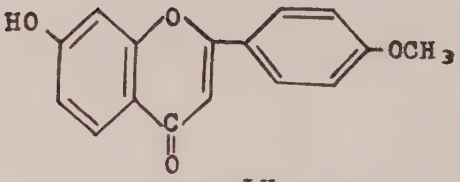
The decomposition of BOA (XLI) and MBOA (XLIII) has been studied.[177] When. *F. nivale* was grown in an oat-glycerol medium in the presence of BOA a pronounced lag period was observed which was longer the higher the BOA content. About 20-30 hr. were required before the decomposition of BOA began and was practically complete after 120 hr. Addition of BOA at this stage showed no lag period indicating that the lag period was possibly the time required for the adaptative formation of BOA decomposing enzyme. Similar results were obtained with MBOA. Spectroscopic evidence suggested the decomposition of these compounds to the corresponding aminophenols. Further, upto a concentration of 0.4 mg./ml. the influence of BOA is fungistatic, at 0.5 mg./ml. a weak fungicidal influence is observed and at concentrations above 0.6 or 0.7 mg./ml. the fungicidal influence prevails.

ISOFLAVONES

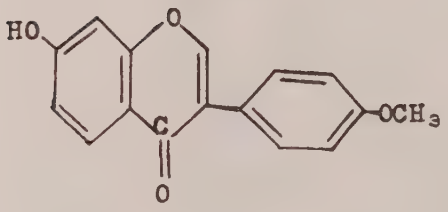
From fresh red clover two crystalline antifungal substances A_1 and A_2 behaving almost alike on paper chromatogram, have been isolated.[162-64] The compound A_1 disappears on drying of the clover. The substance A_2 contains one hydroxyl and one methoxyl group, consumes one mole of bromine in Rossman's method and yields 2,4-dihydroxybenzoic and *p*-hydroxybenzoic acids on alkali fusion and hydrolysis with barium hydroxide gives anisic acid (LIX). On the basis of these results A_2 could be either a 7-OH-4′-methoxyflavone (LX) or the isomeric isoflavone (LXI). The formation of a $C_{15}H_{14}O_4$ ketone (LXII) in alkaline hydrolysis of A_2 definitely indicated A_2 having the isoflavone structure LXI, and this has been confirmed by direct comparison with authentic sample of formononetin.[185]

The isoflavone (LXI) inhibits growth of *Fusaria* in half the concentration (0.02 per cent) and the growth of *Sclerotinia*

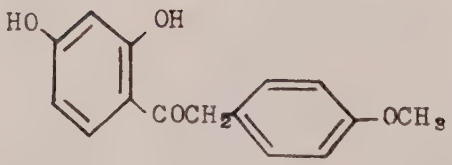
LIX



LX

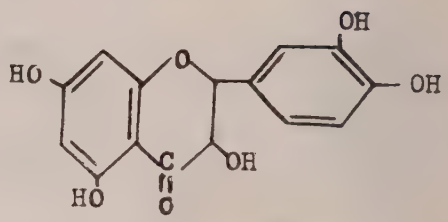


LXI



LXII

in about the same concentration (0.04 per cent) as required by benzoxazolinone.[173] The compound, A_2 is, however, much more active than A_1 against *Sclerotinia* while A_1 has higher activity against Fusaria. The activity of these compounds against Sclerotinia is of considerable interest as *Sclerotinia trifoliorum*, *S. sclerotiorum* and related species are amongst the most destructive fungi that attack a vast variety of plants. Their destructive action is due to the secretion of toxic materials (probably enzymes) which diffuse in advance, kill the protoplasm and soften the cell wall.



LXIII

The heartwood of *Pseudotsuga taxifola* (Douglas fir) contains a flavanolone, taxifolin (LXIII) which has weak fungicidal properties.[122]

ISOTHIOCYANATES

Allyl *iso*thiocyanate (LXIV) was isolated from the seeds of black mustard (*Brassica nigra*) over a hundred years ago. Since then several workers have shown the widespread occurrence of glucosidically bound *iso*thiocyanates particularly in plants

$$CH_2{=}CH\text{-}CH_2\text{-}NCS$$

LXIV

belonging to the family Cruciferae. The older work has been reviewed.[93, 130, 133] Allyl *iso*thiocyanate was considered responsible for the antimicrobial properties of horse raddish (*Alliaria officinalis*) vapours.[61, 62] Its glucoside is termed sinigrin (sinigroside ; potassium myronate). Synthetic methyl *iso*thiocyanate was found more and ethyl derivative less effective than allyl *iso*thiocyanate. Attempts to correlate antifungal activity of *iso*thiocyanates with the disease resistance of plants showed that the toxicity decreased in the order of allyl, phenyl, methyl, and ethyl. The activity of the isomeric compound sometimes varied greatly.[179] Studies on the effects of allyl *iso*thiocyanate and β-phenethyl *iso*thiocyanate on the germination and virulence of the club root organisms indicated that these inhibited the germination of spores of *P. brassicae*. However, at concentrations below the toxic

levels both oils were stimulatory to the germination of spores. *Plasmodiophora brassicae* and *Colletotrichum circinans* spores were inhibited at 0.01 to 0.08 mg./ml. of the allyl and 0.005 to 0.08 mg./ml. of phenethyl *iso*thiocyanates.[79]

The *iso*thiocyanates are easily converted to the corresponding thioureas in the presence of ammonia. A paper chromatographic procedure for the separation and identification of thioureas was developed by Kjaer and Rubinstein.[56, 68, 91, 92]

The *iso*thiocyanates are liberated from their glucosidic precursors, by treatment with a cell free myrosinase preparation.[115] The liberated *iso*thiocyanates are steam distilled and passed into concentrated ammonia whereby the thioureas formed are separated and identified by paper chromatography. The volatile *iso*thiocyanates in seeds and roots of various species of Brassica and in seeds of several other plants are given in Table I.[86,93,94] According to paper chromatographic evidence allyl *iso*thiocyanate occurs most abundantly. Among other small molecular weight *iso*thiocyanates 3-butenyl, *sec*butyl, benzyl, methyl, β-phenylethyl and *iso*propyl have also been isolated.

TABLE I

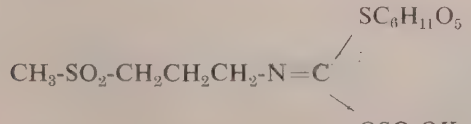
CONTENTS OF VOLATILE ISOTHIOCYANATES (R-N=C=S) IN SEEDS DETERMINED BY PAPER CHROMATOGRAPHY

Species	R	mg. %
Brassica nigra Koch (Black mustard)	Allyl	518
B. juncea Czern. et Coss. (Indian mustard)	Allyl	353
B. pseudojuncea	Allyl 3-Butenyl	470
B. oleracea L. (Cabbage)	Allyl 3-Butenyl	125
B. oler. capitata var. *alba* L. (White cabbage)	Allyl *sec.* Butyl (?)	311
B. oler. capitata var. *rubra* L. (Red cabbage)	Allyl Benzyl (?)	221
B. oler. var. *percrispa* (Kale)	Allyl	8
B. oler. var. *botrytis* L. (Cauliflower)	Allyl *sec.* Butyl	270
B. oler. var. *gemmifera* D. C. (Brussels sprouts)	Allyl *sec.* Butyl	192
B. oler. var. *asparagoides* D. C. (Broccoli)	Methyl (?) Benzyl unidentified	
B. oler. var. *sabauda* L. (Green savow)	Allyl *sec.* Butyl	279
B. napus L. (*rapifera*) *esculenta* D. C. (Kohlrabi)	None	

Species	R	mg. %
B. rapa rapifera Metzger (White turnip)	Unidentified	
B. rapa (*rapifera*) *cummunis* Metzger (Turnip)	3-Butenyl Unidentified Unidentified	2-3
Brassica rapus L. (Rapeseeds)	3-Butyl Unidentified Unidentified	
Cruciferae		
Barbarea arcuata (Opiz.) Reichb.	Methyl β-phenethyl	20 70
B. intermedia Bor.	β-phenethyl	43
Cakile maritima Scop.	Allyl	
Cardamine graeca L.	3-Butenyl Benzyl	591
Cheiranthus cheiri L.	Methyl *iso*propyl	7 11
C. maritimus	Methyl *sec*-Butyl	123
Cochlearia anglica (L.) Asch. and Grb.	*iso*propyl *sec*-Butyl	282 326
C. danica L.	*iso*propyl *sec*-Butyl	220 254
C. officinalis L.	*iso*propyl *sec*-Butyl	254 292
Coronopus didymus (L.) Sm.	Benzyl	154
Crambe maritima L.	Allyl	
Diplotaxis muralis (L.) D. C.	Allyl	500
Draba borealis D. C.	*sec*-Butyl Benzyl (?)	770
D. incana L.	Allyl 3-Butenyl	690
Erucastrum gallicum (Wilde) O. E. Schulz	Allyl 3-Butenyl	34 36
Erysimum cheiranthoides L.	Allyl	8
E. perofskianum Fisch. et May	Methyl Benzyl (?)	179
Hutchinsia alpina R. Br.	β-phenethyl (?)	430
Isatis tinctoria L.	3-Butenyl	
Lepidium densiflorum Schrad.	Benzyl	203

Species	R	mg.%
L. virginicium L.	Benzyl	762
L. sativum. L.	Benzyl β-phenethyl	942
Lunaria annua L.	*iso*propyl *sec*-Butyl	488
Matthiole annua R. Br.	Methyl *iso*propyl	
M. fenestralis (L.) R. Br.	Methyl	
Nasturtium officinale R. Br.	β-phenethyl	558
Raphanus sativus L. var *alba* D. C.	Methyl *iso*propyl	4
R. sativus L. var *radicula*	Allyl	3
Rapistrum perenne (L.) All.	3-Butenyl	
Sinapis alba L.	*iso*propyl	3
Sisymbrium sophia L.	Allyl	30
S. strictissimum L.	*iso*propyl *sec*-Butyl	158
Rasedaceae		
Reseda ungnonette odorata	Methyl	7
Tropaeolaceae		
Tropaeolum majus L.	Benzyl	970
T. majus nanum	Benzyl	977
T. peregrinum (*canariense*)	*iso*propyl *sec*-Butyl	37 43
Capparidacea		
Cleome arabica (L.)	Methyl	30
Gyandropsis gynandra (L.) Briq.	Methyl	251

From the seeds of wall flower (*Cheiranthus cheiri* L.) a crystalline isothiocyanate glucoside called glucocheiroline (LXV) has been reported.[134] The aglucone, cheiroline was shown to be a methyl propyl sulphone (LXVI) both by degradation and synthesis.[132]



LXV

LXVI

An improved synthesis of LXVI in a good overall yield has been reported recently, using acrylonitrile (LXVII) as the starting material.[95]

$$CH_2=CH\text{-}C\equiv N \xrightarrow{CH_3SH} CH_3\text{-}S\text{-}CH_2\text{-}CH_2\text{-}C\equiv N \xrightarrow{LiAlH_4} CH_3\text{-}S\text{-}CH_2CH_2\text{-}CH_2\text{-}NH_2$$

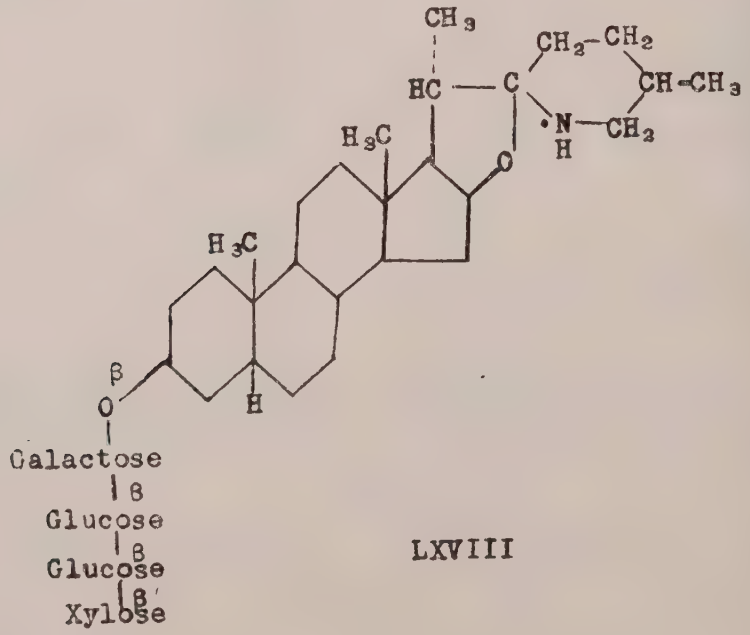
LXVII

$$\xrightarrow{KMnO_4} CH_3SO_2CH_2CH_2CH_2NH_2 \xrightarrow[NaOH]{CSCl_2} LXVI$$

Kjaer and coworkers have isolated a large number of *iso*thiocyanates as their glucosides from plants during the last eight years. Although most of these compounds have not been screened so far for their antimicrobial activity the presence of the common *iso*thiocyanate group and other structural features strongly suggest that the *iso*thiocyanates liberated from these glucosides should possess antifungal properties. This information on their antimicrobial properties would be of great interest.

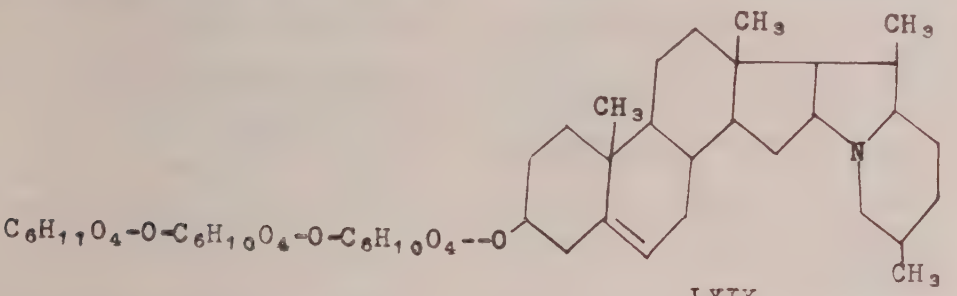
ALKALOIDS

Tomatine (Lycopersicin) (LXVIII) a crystalline glycosidal alkaloid with inhibitory activity against several fungi pathogenic to plants and animals has been isolated from tomato plants.[58, 59, 82, 83, 108, 109] Tomatine contains an aglycone tomatidine and a tetrasaccharide moiety consisting of xylose, galactose, and two glucose units. The aglucone portion tomatidine has also been isolated separately from the roots of Rutgers tomato plant, *Lycopersicum esculentum* (L). Mill.[27]



LXVIII

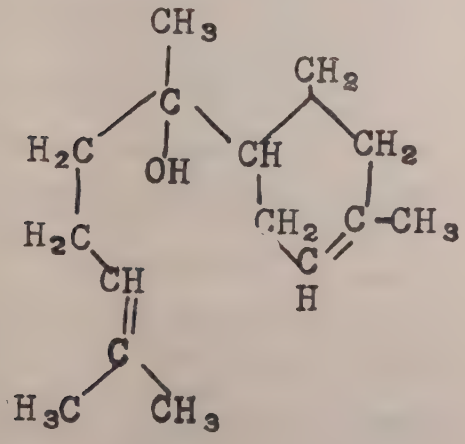
Solanine (LXIX), another alkaloid isolated from several species of Solanum, especially *S. tuberosum* (potato), *S. nigrum*, (woody nightshade), and *Lycopersicum esculentum* (L) Mill., has antifungal activity similar to that of tomatine. Solanine consists of a trisaccharide moiety linked to one mole of its aglycone, solanidine.[18, 26, 59, 126]



LXIX

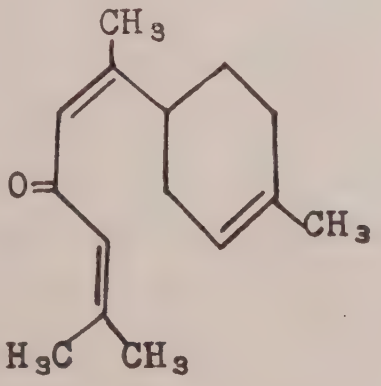
SESQUITERPENES

The neutral oil obtained by ether extraction of buds of *Populus tacamahaca* yielded on purification by distillation and chromatography two sesquiterpenes with *in vitro* activity against *M. tuberculosis* H_{37}. One of the compounds has been identified as ∝-*d*-bisabolol (LXX) and reported active against *Blastomyces dermatitidis*, *Histoplasma capsulatum* at concentrations of about 3 p. p. m.[47]



LXX

Atlantone (LXXI), crude preparations of which have been claimed to have fungicidal properties, is a sesquiterpene contained in the steam distillates of woods of deodar, *Cedrus deodara* (Roxb.) Lond. and the Atlas cedar, *Cedrus atlantica* Maneth.[123]



LXXI

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Studies on the Effect of Streptomycin Spray on the Nodulation and Rhizosphere Microflora of two Green Manure Plants

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STREPTOMYCIN, is one of the antibiotics in use for plant disease control, chiefly against bacterial diseases of plants.[1-4] With a view to examine the possible effects of streptomycin spray on the nodulation and rhizosphere population of two of the common leguminous green manure plants, *viz.*, daincha (*Sesbania aculeata* Pers.) and sunnhemp (*Crotalaria juncea* Linn.), studies were undertaken in this laboratory and the results are presented here. Both these plants are known to form profuse root nodules in symbiosis with *Rhizobium* sp., of the cowpea cross inoculation group, and fix nitrogen. The isolates of *Rhizobium* from these two plants, when tested against streptomycin sulphate by the usual agar dilution streak assay method were found to be inhibited at about 100 μg/ml. of the medium.

MATERIALS AND METHODS

The plants were raised in twelve inch earthenware pots. Twenty-five seeds were sown in each pot and after germination and establishment they were thinned out to retain only 12 plants per pot. The plants were first sprayed 15 days after sowing with aqueous streptomycin sulphate (E. R. Squibb and Sons, containing 729 units/g.) at 250 and 500 p.p.m. concentrations together with 1 per cent glycerine added to improve absorption.[4] In order to prevent any sprayed streptomycin reaching the roots through the soil, the soil surface around the plant was covered with a thick cardboard. The absorption and translocation of the antibiotic in the plant tissues were tested by collecting at periodical intervals representative samples of the leaves, stem and root and plating in the agar media impregnated with a culture of *Bacillus subtilis*, using the tissue culture, paper disc and leaf disc assay methods.

To study the effect of streptomycin spray on the nodulation, sprayed as well as control plants were examined at monthly intervals. One set of plants from a pot was pulled out each time, the nodules on each plant counted and the average number of nodules per plant calculated. To study the rhizosphere population of the plants, samples of root tips were collected at periodical intervals after spraying. The superfluous soil sticking to the root was removed by gentle tapping, the bits were then transferred to 100 ml. sterile tap water and shaken thoroughly. The suspension was then assayed for microbial population by the usual dilution plate technique, using soil-extract and Ken Knight's agar media. There were triplicates in each set of media and sample. On plating the dishes were incubated at room temperature for 7 days, by the end of which the final counts of bacteria, fungi and actinomycetes were recorded. The original rhizosphere suspension was evaporated to dryness on a water-bath and the dry weight of the sample was obtained. The total microbial population per gram of sample on dry weight basis was calculated. For comparison, soil samples collected from unsown pots were also assayed for the microbial population.

EXPERIMENTAL RESULTS

Absorption and translocation of antibiotic

The samples of leaf, stem and root tissues collected at the intervals of 24, 48 and 72 hr. after spraying were assayed for the presence of streptomycin. The results obtained are summarized in Table I.

TABLE I

ABSORPTION AND TRANSLOCATION OF STREPTOMYCIN IN TWO GREEN MANURE PLANTS

Plant samples collected – hr. after spraying	Sprayed with 250 p. p. m. of streptomycin					Sprayed with 500 p. p. m. of streptomycin				
	Cotyledon	Leaf	Stem	Root	Nodule	Cotyledon	Leaf	Stem	Root	Nodule
DAINCHA										
24	+	+	+	—	—	+	+	+	—	—
48	+	+	+	—	—	+	+	+	—	—
72	+	+	+	—	—	+	+	+	—	—
96	+	—	+	—	—	+	+	+	—	—
120	+	—	—	—	—	+	—	—	—	—
168	+	—	—	—	—	+	—	—	—	—
192	—	—	—	—	—	—	—	—	—	—
SUNNHEMP										
24	+	+	+	—	—	+	+	+	—	—
48	+	+	+	—	—	+	+	+	—	—
72	+	+	+	—	—	+	+	+	—	—
96	+	+	+	—	—	+	+	+	—	—
120	+	+	+	—	—	+	+	+	—	—
168	+	—	+	—	—	+	+	+	—	—
192	—	—	—	—	—	—	—	—	—	—

\+ : indication for the presence of streptomycin as seen by the inhibition of *B. subtilis* in culture plates by the paper disc, tissue culture, and agar cup methods.

— : No indication for the presence of streptomycin.

Indications for the presence of streptomycin in the cotyledon, leaves and stem of sunnhemp and daincha were obtained in the samples collected after 24 hr. as well as after 48 and 72 hr., but not in the roots or root nodules. There were no apparent symptoms of adverse effects on the foilage due to the sprays.

Effect on nodulation

The nodulation in the sprayed and check plants was examined at 30 day intervals, as described under Materials and Methods.

The results clearly indicated that there was no significant difference in the number as well as size of the nodules produced in any of the treated plants as compared to the check plants.

Effect on the rhizosphere microflora

The rhizosphere and the soil microflora were assayed at periodical intervals and the results obtaind are summarized in Table II.

TABLE II

THE RHIZOSPHERE POPULATION OF TWO GREEN MANURE PLANTS AS INFLUENCED BY THE STREPTOMYCIN SPRAY

(*Population in* 10^5*/mg. of sample on dry weight basis*)

Treatment and the sample assayed	Before spraying	7 days after spray	14 days after spray	21 days after spray	28 days after spray
DAINCHA					
Bacteria					
(a) 500 ppm strep. + 1% glycerine ..	1197.0	1444.4	1478.5	1459.0	1035.5
(b) Unsprayed check	1307.5	1428.2	1605.0	1384.0	1322.9
(c) Soil	5.1	3.1	7.8	7.9	6.4
Actinomycetes					
(a) 500 ppm strep. + 1% glycerine ..	14.9	9.9	9.3	17.9	29.6
(b) Unsprayed check	19.0	11.4	9.3	12.4	19.3
(c) Soil	1.7	2.4	2.1	3.5	4.1
Fungi					
(a) 500 ppm strep. + 1% glycerine ..	1.7	1.4	1.8	1.5	1.0
(b) Unsprayed check	2.0	1.2	1.0	1.2	0.9
(c) Soil	0.4	0.3	0.4	0.3	0.5
Total population					
(a) 500 ppm strep. 1% glycerine ..	1213.6	1455.7	1489.6	1478.4	1066.1
(b) Unsprayed check	1328.5	1440.8	1615.3	1397.6	1342.7
(c) Soil	7.2	5.8	10.3	11.7	11.0
SUNNHEMP					
Bacteria					
(a) 500 ppm strep. + 1% glycerine ..	1337.3	1460.3	1236.5	1264.5	1283.0
(b) Unsprayed check	1311.5	1663.2	1148.0	1341.0	1323.8
(c) Soil	5.1	3.1	7.8	7.9	6.4
Actinomycetes					
(a) 500 ppm strep. + 1% glycerine ..	10.2	16.8	15.8	15.2	29.7
(b) Unsprayed check	8.7	11.0	14.4	24.6	32.0
(c) Soil	1.7	2.4	2.1	3.5	4.1
Fungi					
(a) 500 ppm strep. + 1% glycerine ..	2.0	1.3	1.3	1.6	1.0
(b) Unsprayed check	1.9	1.4	1.1	0.9	1.5
(c) Soil	0.4	0.3	0.4	0.3	0.5
Total					
(a) 500 ppm strep. + 1% glycerine ..	1344.5	1478.4	1253.6	1281.3	1313.7
(b) Unsprayed check	1322.1	1675.6	1164.5	1366.5	1357.3
(c) Soil	7.2	5.8	10.3	11.7	11.0

The microbial population in the rhizosphere region was considerably more than that of the soil. In daincha the increase in population was : bacteria 175 to 461 times, actinomycetes 3 to 11 times, fungi 2 to 5 times and the total population 119 to 248 times. In sunnhemp the increase was : bacteria 147 to 554 times, actinomycetes 4 to 8 times, fungi 2 to 5 times and total population 113 to 264 times. The streptomycin-glycerine sprays, did not significantly alter the population ranges in the rhizospheres of the green manure plants. This was also confirmed when the results were statistically analysed.

DISCUSSION

One of the important qualities of antibiotics as agents for controlling plant diseases is their systemic translocation in plants. The systemic translocation of antibacterial antibiotics in leguminous plants, when they reach the root system is likely to inhibit the beneficial nodule formation, provided the antibiotic is inhibitory to the *Rhizobium* sp. concerned and also the concentration reaching the root is strong enough for the inhibition. When potassium gibberellate was sprayed on dwarf beans (*Phaseolus vulgaris* L.) it was found to inhibit nodule formation in the root.[5-7] In the present studies, however, it was found that not only the antibiotic sprayed on the foliage could not be traced in the root system but also it did not inhibit or enhance the nodule formation. This is in conformity with the results obtained by other workers on beans.[4,8]

It is quite probable that the presence of a chemical substance in plant tissues may alter its physiology and growth and materially alter the rhizosphere population.[9,12] In the present studies, however, no evidence could be obtained for any drastic change in the quantity of rhizosphere population due to streptomycin-glycerine or glycerine spray indicating thereby that the physiology of the plants was not materially altered. It was, however, revealed that the rhizosphere region of *S. aculeata* and *C. juncea* encourage better growth of bacteria than the fungi and actinomycetes. These results are in conformity with the reports that the legumes support better rhizosphere populations, especially bacteria, than nonlegumes.[11]

SUMMARY

The translocation of streptomycin in two green manure plants, *Sesbania aculeata* Pers. and *Crotalaria juncea* Linn., was studied and it was found that in the cotyledon, stem and leaves of the plants the antibiotic could be detected 24 hr. after spraying, but could not be traced in the tap roots, lateral roots and root nodules even 72 hr. after spraying. There was no significant difference in the nodulation and rhizosphere population of the plants. The rhizosphere population in *S. aculeata* was: bacteria 175 to 461 times, actinomycetes 3 to 11 times and fungi 2 to 5 times than that of soil and in *C. juncea* the population was : bacteria 147 to 554 times, actinomycetes 4 to 8 times and fungi 2 to 5 times than that of the soil, indicating thereby that the two plants induced better growth of the bacteria than the other microorganisms in the rhizosphere region.

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Hamycin in the Treatment of Seborrheic Dermatitis of the Scalp

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SEBORRHEIC dermatitis or *Pityriasis capitis* popularly known as dandruff is a common type of dermatosis of the scalp associated with abundance of oil secretion. The scales of the dandruff when examined under the microscope consistently reveal the presence of the yeast organism *Pityrosporum ovale*. It has been the object of several detailed studies by mycologists and dermatologists. The organism was found to be quite fastidious in its nutritional requirements, and several investigators have successfully cultured the fungus *in vitro*. Benham [2, 3] showed that it is a lipophilic fungus, its growth being enhanced by addition of thiamin and oxaloacetic acid. Spoor *et al.*, [12] showed that the organism is universally present and in artificial culture manifests considerable pleomorphism.

From the point of view of the dermatologist, the role of *P. ovale* as the chief incitant of seborrheic dermatitis or dandruff has become the subject of a great deal of discussion. Though Sabouraud[7] firmly believed that the cause of *Pityriasis simplex* or dandruff was due to infection by *P. ovale*, many of the later workers like Whitlock[13] have disputed this.

While the aetiology of dandruff is debated upon, fungicidal substances have been employed for the treatment of dandruff with good results. For several centuries, sulphur has been the drug of choice, in combination with resorcin or salicylic acid. These preparations had their own disadvantages in that they were greasy and inconvenient to use. Recurrence of the diseased condition after the discontinuation of treatment was quite frequent, and the odour of sulphur was disagreeable to most of the patients.

In recent years selenium disulphide [8, 10, 11] and cadmium sulphide [6] have been used in the treatment of dandruff. Though selenium disulphide is quite toxic, topical application of 2.5 per cent solution for 5 to 10 min. as shampoo has been considered not dangerous, since there is very little absorption.

In the present paper, the results of treatment of seborrheic dermatitis of the scalp with the antifungal antibiotic Hamycin* is presented. Very effective control of dandruff was obtained in all the cases treated, which pointed out the need for revising our concept about the aetiology of dandruff. A brief account of the experiments carried out is presented here.

MATERIALS

Material : A total of forty-eight cases consisting of twenty male and twenty-eight female patients were studied. Their ages ranged from 8 to 45 years. The cases under trial were divided into two groups according to clinical symptoms, *Pityriasis sicca* and *Pityriasis oleosa* (Table I).

* Hamycin is an antifungal antibiotic manufactured by Hindustan Antibiotics Ltd., Pimpri, Poona.

TABLE I

Pityriasis sicca			*Pityriasis oleosa*		
Male	Female	Total	Male	Female	Total
12	20	32	8	8	16

Out of these 48 cases, 8 cases (4 of *P. sicca* and 4 of *P. oleosa*) were kept as controls. These were treated with blank solutions (50 per cent alcohol solution without the antibiotic). Remaining 40 cases were divided into two groups, the first group of 28 persons consisting of *P. sicca* type and second group of 12 persons with *P. oleosa* type of infection (Table II).

TABLE II

	Control cases treated with blank solution	Cases treated with Hamycin solution
P. sicca	4	28
P. oleosa	4	12
Total	8	40

METHODS

(1) A 0.05 per cent Hamycin solution was prepared in 50 per cent ethyl alcohol.

(2) Control cases were treated with 50 per cent alcoholic solution without Hamycin.

(3) The patients were required to apply about 10 ml. of the solution, with good rubbing, twice a day. During the period of treatment they were advised not to use oil over the scalp.

(4) Clinical check up was done after every 7 days.

RESULTS

1. P. sicca group

After the first week of treatment, in 25 patients out of 28 of this group there was a marked decrease in size and number of scales, which became dry and brittle. Similar effects were not observed in the remaining 3 cases of this group. Later it was found that these 3 patients had used vegetable oil during the period of treatment.

Microscopic examination of the scales in potassium hydroxide solution revealed a consistent fall in number of cells of *P. ovale* with the progress of Hamycin treatment as compared with the controls. Similar results were obtained in the three other cases mentioned above only after 12 days. The twenty-five cases became completely free of scales after 12 days ; the three cases wherein the patients initially continued to use oil, became free of scales after 18 days. All the patients were asked to continue the application of Hamycin solution for one more week after the clinical cure. No side effects of any kind were noted during the treatment or later on. All the 28 cases were followed up for a month after the completion of the therapeutic course. Mild type of scaling possibly due to reinfection was noted in 5 patients only.

2. P. oleosa group

The response in this series was comparatively slower than in the *P. sicca* group. A marked decrease in scaling was observed in six cases after 20 days and in the remaining six cases after 25 days. Two cases showed a mild scaling even after four weeks treatment. Remaining 10 cases showed no relapse.

Control cases.

Eight cases (4 cases of *P. sicca* and 4 of *P. oleosa* type) treated with the blank solution (50 per cent ethyl alcoholic solution without the antibiotic) showed no change in the number of scales or degree of infection except that the scalp became dry due to application of alcohol.

DISCUSSION

From the studies made so far it is clear that the antifungal antibiotic Hamycin gives an effective clinical cure for Seborrhoea capitis. Selenium disulphide shampoo has been reported to be effective against dandruff by many workers, but the fact that treatment is often associated with side effects, needs careful consideration. Bereston[4] has reported excess of oiliness in 31 per cent of the cases, and development of orange tint in 19 per cent of patients with grey hair due to selenium disulphide shampoo. Sidi and Spinasse[9] have observed harmful effects of selenium sulphide on hair in cases of alopecias and pityriasis. Partial loss of hair has been reported by Grover.[5] Weakening of hair roots and breaking of hair in the shaft has been recently pointed out by Archer and Luell.[1]

Treatment with Hamycin was found to be safe and there were no adverse effects in the cases treated. The application of the solution is quite convenient. In general, *Pityriasis sicca* cases respond better than *P. oleosa* cases.

SUMMARY

Hamycin, has been found to be effective in the treatment of Seborrheic dermatitis. For the treatment of dandruff, Hamycin was used as 0.05 per cent solution in 50 per cent ethyl alcohol. Ten ml. of the solution was applied to the scalp by massaging, twice a day. Twenty-eight cases with *Pityriasis sicca* type infection showed complete clinical cure after 12 to 18 days of treatment. Twelve cases of *Pityriasis oleosa* type that were treated with the antibiotic took slightly longer time for complete clinical cure. Controls that were treated with 50 per cent alcohol only, showed no reduction in the amount of scales. There were no side effects of any kind or recurrence of infection in the cases treated with the antibiotic.

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Oxidative Metabolism of *Penicillium chrysogenum*

I. OXIDATION OF CARBOHYDRATES

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KNOWLEDGE of oxidative metabolism of the penicillin producing mould *Penicillium chrysogenum* has been more or less fragmentary and derived mainly from experiments in shaken flasks and laboratory fermentors, and has little reference to conditions prevailing during production of penicillin in commercial fermentors. Due to high endogenous respiration peculiar to fungi and the permeability barriers in these organisms, investigations on the oxidative metabolism of the intact mycelial cells by manometric technique have been unsatisfactory and many workers have found it difficult to demonstrate the stimulatory effect of an added substrate to freshly washed intact mycelial cells. Studies on oxidative metabolism of *P. chrysogenum* have, therefore, been confined to the use of cell-free extracts and radioactive tracer substances.

In the present series of papers, investigations on the oxidation of various substrates by a commercial strain of *P. chrysogenum* HA-9 (a derivative of a Russian strain) grown under penicillin production conditions are reported. The conventional manometric technique has been suitably adapted to demonstrate the effect of added substrates.

Work on carbohydrate metabolism of *Penicillium chrysogenum* has been mostly devoted to the study of different pathways of breakdown of carbohydrates, especially glucose. A study of enzyme systems in cell-free extracts of the mould by Knight and coworkers, [1, 2] and investigations using radioactive tracer substances by Koffler and coworkers[3-6] have shown the occurrence of both the Embden-Meyerhof-Parnas and hexosemonophosphate pathways for the breakdown of carbohydrate in *P. chrysogenum*. Results of a study of oxidation of various carbohydrates by mycelium of *P. chrysogenum* obtained from production fermentors during peak hours of penicillin biosynthesis are recorded in this paper.

MATERIALS AND METHODS

Mycelial samples were collected fresh from 5,000 gallon fermentors between 45-50 hr. of fermentation. The fermentation medium employed was the usual cornsteep-lactose medium with 2 per cent lactose initially and sucrose feeding from 48 hr. onwards. Rest of the fermentation conditions were the same as reported in an earlier communication.[7]

For manometric study the following four different cell preparations were used :

1. Fresh intact cells

About 3.0 ml. of mycelial sample was washed thrice with distilled water in a 15 ml. centrifuge tube, every time suspending the packed cells in fresh water, and finally suspended in about 12 ml. of 0.1 *M* phosphate buffer, pH 7.0. One ml. of this suspension was used for each Warburg flask (dry weight of mycelium, 3.5-4.0 mg./flask).

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2. **Fresh blenderized cells**

About 15.0 ml. of mycelial sample was filtered over a nylon cloth and squeezed gently to remove as much of adhering broth as possible. It was then washed three times with water and finally suspended in 50 ml. of cold 0.1 *M* phosphate buffer, pH 7.0, and blenderized in a previously cooled Waring blender at +5° for 30 sec. One ml. of this suspension was used for each Warburg flask (dry weight of mycelium, 3.5-5.5 mg./flask).

3. **Pre-starved mycelial cells**

About 25.0 ml. of mycelial sample collected under aseptic conditions were filtered over a nylon cloth and thoroughly washed with sterile distilled water as described above. The washed mycelium was then resuspended in 50 ml. of sterile 0.1 *M* phosphate buffer, pH 7.0 in a 500 ml. Erlenmeyer flask and placed on a rotary shaker (240 r.p.m., 2″ throw) for 24 hr. at 24°. The pH of the suspension was unaltered at the end of the starvation period. About 15.0 ml. of this starved mycelial sample was centrifuged, and the packed cells resuspended in 15 ml. of fresh 0.1 *M* phosphate buffer pH 7.0. One ml. of this suspension was used for each Warburg flask (dry weight of mycelium, 7·0-7.5 mg./flask).

4. **Fresh intact cells at pH** *2.6*

About 10 ml. of the sample was washed with distilled water in a centrifuge, as described in procedure (1) above. The washed mycelium was suspended in about 15 ml. of 0.1 *M* phthalate-HCl buffer, pH 2.6. One ml. of this suspension was used for each Warburg flask (dry weight of mycelium, 13.0-15.0 mg./flask).

Dry weight of the cells was determined as described earlier[7] using suitable aliquot from the final cell suspensions employed for manometric study.

Oxygen uptake was measured by the conventional manometric technique[8] using a refrigerated circular Warburg apparatus (American Instruments Co.) at 24°. The system consisted of 1.5 ml. 0.1 *M* buffer and 1.0 ml. of cell suspension in the main compartment of a 15 ml. Warburg flask ; 0.2 ml. of 5*N* KOH was taken in the centre well to absorb CO_2 and 0.5 ml. of 0.1 *M* substrate solution (corresponding to 50 (M) was μtipped from the side arm.

Oxygen consumption was measured for a period of 5 hr. in case of cell suspensions at pH 7.0, and for a period of 2 hr. with cells suspended in pH 2.6 buffer. The measurements were made every 10 min. for the first half hour, and then at 30 min. intervals till the end of the experiment.

Oxidation of the following carbohydrates was studied :

Pentoses: D(—) arabinose, L(+) arabinose, and D(—) ribose (Eastman).

Hexoses: D-glucose (J. T. Baker Chemical Co.), fructose, D(+) mannose, (D+) galactose, L(—) rhamnose and L(—) sorbose (Eastman).

Disaccharides: Lactose (Judex Chemicals), sucrose (E. Merck), D(+) maltose, cellobiose and melibiose (Eastman).

Oligosaccharide: raffinose (Eastman).

Polysaccharides: Inulin (Thomas Kerfoot and Co.) and starch soluble (E. Merck).

RESULTS

Intact mycelium at pH *7.0*

Table I shows the results of oxidation of various carbohydrates by fresh intact mycelial cells at pH 7.0. Most of the sugars were found to be oxidized after a lag period of about 2 hr. At the end of fifth hour total oxygen uptake was well

over 50 per cent higher than endogenous in presence of glucose, mannose, maltose, sucrose, cellobiose, melibiose, raffinose and sorbose, 35-40 per cent higher in presence of fructose, rhamnose, galactose and L(+) arabinose. Ribose and starch were very slowly oxidized. Lactose, D(—) arabinose and inulin did not produce any stimulation of oxygen uptake even after 5 hr. of incubation.

TABLE I

OXIDATION OF CARBOHYDRATES BY *Penicillium chrysogenum* (STRAIN HA-9)

(*Fresh intact mycelium at pH 7.0, temp. 24°*)

	μl oxygen uptake/mg. dry weight				
Hours	1	2	3	4	5
Substrate*					
Expt. 1					
None	22.2	38.9	53.2	63.9	74.7
D-Glucose	23.6	47.7	70.9	94.5	117.3
Fructose	21.8	42.4	63.8	84.2	103.5
D(+) Galactose	24.0	47.4	67.9	88.0	107.0
D(+) Mannose	25.6	48.4	70.8	94.0	117.2
D(+) Maltose	22.8	46.4	70.0	93.2	116.8
Lactose	20.8	38.4	53.6	67.6	79.7
Sucrose	25.3	50.9	74.9	99.7	123.3
Expt. 2					
None	21.6	37.2	49.9	59.7	68.5
D(—) Arabinose	21.5	37.8	52.4	63.6	73.5
L(+) Arabinose	21.3	41.6	58.9	76.1	92.9
Cellobiose	24.1	47.4	71.2	96.7	123.4
Melibiose	24.4	46.6	68.4	90.8	114.1
Raffinose	21.0	40.8	60.6	86.3	108.9
L(—) Rhamnose	21.4	40.1	57.0	74.2	93.8
D(—) Ribose	20.1	36.3	52.3	66.6	83.2
L(—) Sorbose	20.8	39.8	60.1	81.8	104.9
Inulin	20.0	39.7	53.0	58.8	68.2
Starch	21.6	38.7	53.7	68.0	81.9

All figures represent average of duplicate determinations varying within 0-5 per cent.

* Substrate added was 50 μM per flask.

Blenderized mycelium at pH *7.0*

Results with blenderized mycelium were similar to those with intact cells, the response to added sugars being slightly higher (Table II).

TABLE II

OXIDATION OF CARBOHYDRATES BY *Pencillium chrysogenum* (STRAIN HA-9)

(*Fresh blenderized mycelium at pH 7.0*, temp. *24°*)

	μl oxygen uptake/mg. dry weight				
Hours	1	2	3	4	5
Substrate*					
Expt. 1					
None	13.3	21.3	28.3	33.5	37.1
D-Glucose	13.0	24.7	36.1	48.1	61.1
Fructose	10.9	20.6	30.5	40.4	49.8
D(+) Galactose	9.8	20.5	31.2	41.6	53.3
D(+) Mannose	12.0	22.7	34.1	46.1	58.1
Lactose	12.4	21.4	29.8	37.1	42.5
D(+) Maltose	13.1	25.1	36.8	49.6	62.0
Sucrose	13.0	23.8	35.4	47.4	60.1
Expt. 2					
None	16.0	27.8	37.2	44.8	51.0
D(—) Arabinose	15.4	27.6	37.3	45.0	51.9
Cellobiose	17.1	32.3	44.0	56.8	70.2
Melibiose	17.0	33.4	48.9	64.4	84.7
Raffinose	17.1	33.0	48.5	63.6	77.9
L(—) Rhamnose	16.7	30.2	42.1	53.2	63.1
D(—) Ribose	16.9	31.6	43.1	53.4	62.0
L(—) Sorbose	16.2	32.9	48.5	64.5	80.9
Inulin	15.6	27.5	37.0	44.9	51.7
Starch	16.0	28.7	38.0	48.2	59.2

All figures represent average of duplicate determination varying within 0-5 per cent.

* Substrate added was 50 μM per flask.

Pre-starved mycelium at pH *7.0*

On account of partial utilization of endogenous substrates in prestarved mycelium, the response to added sugars was more readily elicited (Table III). With the exception of D(—) arabinose and inulin the rest of the sugars showed measurable stimulation of oxygen uptake even within the first hour of incubation ; lactose appeared to be the most slowly oxidized sugar among the lot.

TABLE III

OXIDATION OF CARBOHYDRATES BY *Penicillium chrysogenum* (STRAIN HA-9)

(*24 hr. starved mycelium at pH 7.0, temp. 24°*)

	μl oxygen uptake/mg. dry weight				
Hours	1	2	3	4	5
Substrate*					
Expt. 1					
None	7.7	13.6	18.0	20.5	22.5
D-Glucose	10.0	21.5	34.4	47.3	62.7
Fructose	10.0	20.8	30.9	42.3	54.9
D(+) Galactose	9.4	19.8	30.0	40.3	52.7
D(+) Mannose	9.6	20.8	33.2	45.8	61.5
D(+) Maltose	10.3	23.0	36.7	51.2	68.2
Lactose	8.4	15.5	21.5	27.4	33.4
Sucrose	10.0	21.9	35.4	49.0	66.1
Expt. 2					
D(—) Arabinose	8.7	14.7	19.4	22.7	25.4
L(+) Arabinose	9.1	15.2	20.1	23.7	26.8
Cellobiose	10.5	20.9	32.7	46.8	62.7
Melibiose	11.1	22.7	35.7	50.9	68.9
Raffinose	10.7	21.9	34.1	48.1	65.9
L(—) Rhamnose	9.7	19.8	30.3	42.7	57.5
D(—) Ribose	10.5	21.0	31.6	44.4	59.5
L(—) Sorbose	10.4	21.3	34.0	48.4	65.3
Inulin	8.7	14.7	19.4	22.8	25.6
Starch	10.5	21.4	33.2	47.0	63.2

All figures represent average of duplicate determinations varying within 0-5 per cent.

* Substrate added was 50 μM per flask.

Oxidation at pH *2.6*

It was interesting to observe that oxidation of many of these sugars by intact fresh mycelium could be readily demonstrated if the pH of incubation was made highly acidic (pH 2.6) (Table IV). The rate of endogenous respiration was, however, considerably diminished at pH 2.6, presumably due to partial denaturation of the respiratory enzymes. D(—) arabinose, inulin and starch showed no response at this pH.

TABLE IV

OXIDATION OF CARBOHYDRATES BY *Penicillium chrysogenum* (STRAIN HA-9)

(*Fresh intact mycelium at pH 2.6, temp. 24°*)

	μl oxygen uptake/mg. dry weight	
Hours	1	2
Substrate*		
Expt. 1		
None	3.2	4.6
D-Glucose	4.3	6.7
Fructose	4.5	7.1
D(+) Galactose	4.1	6.6
D(+) Mannose	4.2	6.6
D(+) Maltose	4.6	7.7
Lactose	4.0	6.3
Sucrose	4.5	7.5
Expt. 2		
None	3.6	5.5
D(—) Arabinose	3.7	5.6
L(+) Arabinose	3.8	5.8
Cellobiose	4.2	6.6
Melibiose	4.0	6.5
Raffinose	4.0	6.1
L(—) Rhamnose	3.9	5.9
D(—) Ribose	3.7	5.8
L(—) Sorbose	4.0	6.2
Inulin	3.6	5.3
Starch	3.6	5.5

All figures represent average of duplicate determinations varying within 0-5 per cent.

* Substrate added was 50 μM per flask.

DISCUSSION

From the data presented above it is evident that the simple and conventional manometric technique can be employed to measure oxidation of added substrates provided the incubation period with fresh intact mould mycelium is sufficiently prolonged. Alternatively, pre-starved mycelium or incubation of fresh mycelium at highly acidic pH (pH 2.6) can be successfully used for short term manometric experiments. Transport of substrate across a cell wall is postulated to be mediated through synthesis of adaptive enzymes called permeases [9, 10] which are elicited in response to specific substrates. Such transport mechanism in *P. chrysogenum* appears to be highly specific with regard to optical configuration of arabinose. While L(+) arabinose is readilly oxidized by the mould the D(—) isomer is not touched at all.

Lactose is the sugar of choice in penicillin fermentation on account of its slow utilisation. From estimation of residual lactose during penicillin fermentation it becomes evident that lactose is progressively consumed by the mould at pH 7.0. But with washed mycelium oxidation of lactose cannot be readily demonstrated manometrically unless it is prestarved or incubated at highly acid pH ; even then the rate of oxidation is very slow compared to that of glucose or galactose at neutral pH. It thus appears that the formation of the specific adaptive enzyme (permease) for transport of lactose is a very slow process in *P. chrysogenum* and so also the rate of emzymatic breakdown of lactose to glucose and galactose within the cell.

SUMMARY

Oxidation of seventeen sugars comprising of pentoses, aldo- and keto-hexoses, disaccharides, oligosaccharide and polysaccharides by intact and blenderized mycelial cells of *P. chrysogenum* was studied manometrically. All disaccharides except lactose were quite rapidly oxidized by the mould at pH 7.0. Hexoses, pentoses and starch were oxidized at a slightly slower rate than disaccharides. Lactose was very slowly oxidized by the mould and the effect could be demonstrated only with prestarved cells at pH 7.0, or working at highly acid pH of 2.6. While L(+) arabinose was quite rapidly oxidized, D(—) arabinose was not at all oxidized by the mould. Inulin also was not oxidized by the mould.

Response to added sugars in manometric study was evident only after a period of adaptation for a few hours with fresh intact cells or blenderized cells at pH 7.0, while at low pH (2.6) the oxidation was readily demonstrable.

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Optimization of Fermentation Cycle

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IN the production of penicillin by *P. chrysogenum*, there are three distinct phases in the rate of production in the fermentor proper. There is an initial lag phase, during which the rate of production increases with time and after a period of 24-28 hr. reaches a maximum. In the second phase, the rate remains substantially constant till autolysis of the mycelium sets in. In the last phase, the rate begins to go down.

An attempt is made in this note to determine from purely mathematical considerations the length of the fermentation cycle which will result in the maximum production of penicillin per unit fermentor volume.

Let

x = length of the optimum cycle of fermentation, *i.e.* from the seeding time to harvesting, in hours ;

y = average time required to prepare a fermentor from harvesting to seeding, in hours.

Then, total number of batches (n) per month (of 30 days) per fermentor will be

$$n = \frac{720}{x + y}$$

Also let

a = rate of production at the end of the cycle in activity units per ml. per hour, when

b = the final titre units per ml.

As b will not be a constant for each batch, value of a will vary with b.

Minimum rate of production at the end of the cycle is then given by

$$a = \frac{b}{x + y} \quad .. \quad .. \quad ..(1)$$

Values of a can then be calculated for several values of b and b vs. a plotted as a straight line relationship.

Due to biological variations, all fermentation batches will not follow a definite pattern which will give the same value for optimum cycle. Statistical studies, however, would reveal whether the cycle followed is near optimum conditions. Titre at (x—6) hr. and average rate of production between the period x hr. and (x—12) hr. may be plotted on the straight line relationship obtained above. The deviation of the points referring to various batches may be studied. If the value of x corresponds to optimum conditions, the points will be distributed on both sides of the line to an equal extent, both with regard to number of points and the extent of deviations. If the cycle is too short, a larger number of points will be *above* the line and *vice versa*.

For a more accurate study of the optimum cycle, it may be necessary to take samples more frequently at the end of the cycle and to determine the instantaneous rate of production from the slope of the titre vs. time curve.

From an analytical consideration of equation (1), it will be seen that the maximum rate of production during the second phase will be higher than $\frac{b}{x}$ due to the existence of the initial lag phase. The final rate of production will thus be distinctly less than the highest rate. This is a rough and ready guide to make sure that the cycle is near optimum.

The above reasoning does not take into account any variation in the recovery efficiency with the length of the cycle. It is known that with some strains, an optimum cycle as determined above, may lead to discolouration of the first crystals as also lowering of recoveries. Determination of the optimum cycle for production as above, also does not take into account the incidence of cost of raw materials. If this is taken into account, it will tend to make the optimum cycle even longer. However, when cane sugar or other disaccharide is used to meet the carbohydrate requirements of the mould, it is required to be fed continuously till almost the end of the cycle. The consumption of this expensive raw material is, therefore, dependent upon the length of the cycle and makes the raw material cost less sensitive to the evaluation of overall optimum cycle.

In actual plant practice where fermentors are harvested and treated subsequently as separate batches without any mixing, there are certain limitations to variations in the length of the cycle. Such variations are best made in certain fixed quanta depending upon the number of fermentors available. If the optimum cycle falls wide off the range of such adjustments, the seeding and the harvesting time will be different for each day of the month. It should also be appreciated that due to irregularities in the growth of seed as also in other operational factors, it is not possible to have for all fermentors the same length of cycle. Appropriate corrections should be made when such batches are plotted on the straight line relationship developed above.

The total optimum output from a given gross volume of all fermentors will then depend upon :

(a) The final volume in the fermentor at the time of harvesting.

(b) Percentage recovery in extraction.

(c) Proper determination of optimum cycle, taking into account variations, if any, in recovery with time cycle.

(d) Value of y.

A long-cycle fermentation is desirable both from the point of view of consumption of raw material as also productivity. The value of y tends to have less and less effect on production as x increases.

In order to be sure that the cycle actually followed in plant practice is always optimum, periodic checks should be made using the method described above. Whilst this is essential for all strain selection programme, such checks should also be carried outon the strains in current use because of variations in the conditions such as quality of raw materials (variations in trace elements and other indeterminate factors) and other normal variations in the fermentation and extraction processes.

SUMMARY

A mathematical formula has been derived to estimate the length of the fermentation cycle which will result in the maximum production of penicillin per unit fermentor volume.

SHORT NOTES

Countercurrent Distribution Studies. II. 6-Aminopenicillanic Acid and its Separation from Penicillin G

6-AMINOPENICILLANIC acid (6-APA)[1-3] the nucleus of penicillins, has been obtained in crystalline form in this laboratory by fermentation of *Penicillium chrysogenum* in synthetic medium as well as by enzymatic splitting of penicillin G (Patents pending). Recently, Arnstein *et al.*,[4] detected, by means of paper chromatography, the presence of a compound other than 6-APA in the mycelium of *P. chrysogenum*. This substance resembles 6-APA in that it shows antibacterial activity only when reacted with phenylacetyl chloride at a pH just above neutral point. This prompted us to test the homogeneity of 6-APA by the technique of countercurrent distribution. An attempt was also made to separate 6-APA from its mixture with penicillin G. It deserves mention that any known method of isolation of 6-APA involves its separation from penicillin G or penicillin V.

After several trials *n*-butanol-citrate buffer (pH 6.5) was selected as the solvent system for distribution studies. When distributed alone 6-APA showed a single peak at the 3d tube on a 20-tube transfer (Fig. 1, curve 2). Penicillin G, however, migrated further under identical conditions and maximum concentration was attained at the 15th tube for the same number of transfers. This indicated clearly the possibility of easy separation of 6-APA from penicillin G in the solvent system under investigation. Curve 3 in Fig. 1 shows the resolution of 6-APA and penicillin G from a mixture of both.

Estimations of 6-APA in individual tubes were made by the modified iodometric method in the case of aqueous phase (Curve 2). The assay of 6-APA in the butanol phase was carried out by the spectrophotometric method at 268 mμ on a Beckman spectrophotometer Model DU (Curve 1). As additional proof, aliquot samples (lower phase) from individual tubes upto tube No. 10, as indicated by the iodometric assay,[5] were tested as such for antibacterial actitvity against *B. subtilis* after suitable dilution and also after treatment with phenylacetyl chloride at pH 7.5.[5] Antibacterial activity was demonstrated only after acylation. Samples from tubes 14-20, however, showed antibiotic property even without acylation indicating the presence of penicillin G.

Attempts were made to resolve a crude mixture of penicillin G and 6-APA. For this purpose penicillin G was split up enzymatically at 37^0 for 6 hr. and the mixture centrifuged in order to remove the microbial cells. The solution containing unconverted penicillin G and 6-APA was used as the starting material for distribution studies using the solvent system mentioned above. Curve 4 shows the distribution pattern obtained for a 20-tube transfer. Estimations were made as before by the iodometric method as also by microbiological assay. In some cases tube 0 contained a larger amount of material titrable with iodine, possibly resulting from the deterioration of penicillin G or 6-APA on being kept long during the course of enzymatic reaction. This was suggested by the low value of blank in the iodometric estimation and corroborated by the presence of low antibacterial activity in samples withdrawn from this tube. Further investigations are in progress.

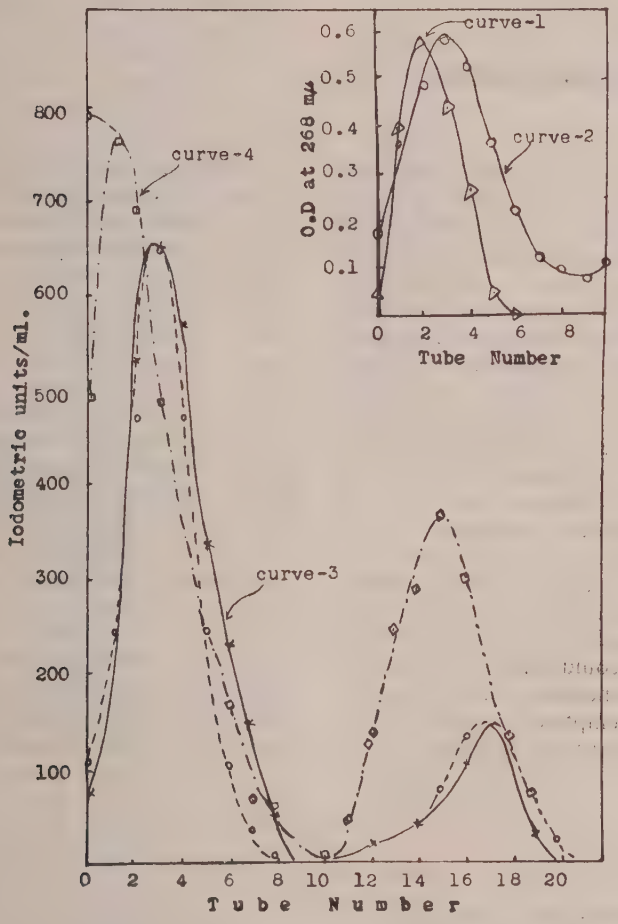

Fig. 1. Countercurrent distribution of 6 — APA
○ — — ○ Theoretical
□ — . — □ Experimental

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Studies on Penicillin Amidase

SAKAGUCHI and Murao[1] first reported that an enzyme present in the mycelium of *Penicillium chrysogenum* could split penicillin G into 6-aminopenicillanic acid (6-APA) and phenylacetic acid. After repeated failures[2] this observation has recently been confirmed by the Squibb group of workers.[3] Meanwhile an extensive survey of different micro-organisms revealed that the enzyme amidase occurs fairly widespread among micro-organisms particularly among *Eubacteriales* of different genera.[2, 4] In connection with general screening of the coli group of bacteria,[4] which was first reported by Rolinson *et al.*[2] to possess the enzyme activity, we came across a strain of *Escherichia coli* (designated as HA-61), which could split penicillin G under suitable conditions. In a separate experiment an attempt was made to isolate the enzyme and purify it suitably.

For this purpose the bacterium was grown in suitable media at 25° for 16 hr. on a rotary shaker, the cells centrifuged in a Sharples supercentrifuge and a suspension of the bacterial cells in 0.1*M* phosphate buffer pH 7.5[4] was sonicated in a MSE ultrasonic disintregrator (10 K.C., 0.8-1.2 amp.). The whole suspension including disrupted cells was centrifuged at 10,000 g. for 1 hr. at $+5^0$ and from the cell-free extract the enzyme was purified by treatment with 0.45 saturated ammonium sulphate and then by acetone precipitation (5-10 vol.) in cold (6-8 fold). Final purification by ion-exchange chromatography over IRC-50 (200 mesh) showed promising results.

To estimate the amount of enzyme present, a manometric method was developed, which is principally based on the measurement of carbon dioxide evolved from

TABLE I

AMIDASE ACTIVITY AT 37°, pH 7.5

		μl CO_2 evolved						
Flask No.		1	2	3	4	5	6	7
Enzyme (ml)		0.6		0.2	0.4	0.6	0.8	1.0
Substrate (ml)					0.5			
1st hr.	uncorrected	+3	+8	+12	+17	+21	+25	+29
	corrected			+6.4	+14.9	+21.5	+28.7	+33.0
2d hr.	uncorrected	+10	+10	+14	+19	+23	+28	+33
	corrected			+6.4	+14.9	+21.5	+30.4	+36.2
3d hr.	uncorrected	+5	+7	+11	+15	+19	+24	+29
	corrected			+6.4	+13.0	+19.8	+28.7	+34.7

Enzyme : Dialysed $(NH_4)_2SO_4$ precipitated fraction.
Substrate : Na Penicillin G, 2 mg./ml.
Atmosphere : 95% N_2 + 5% CO_2 (Flasks gassed for 10 min.)
Buffer : $NaHCO_3$ + CO_2 buffer pH 7.5.

(0.032*M*) bicarbonate buffer (pH 7.5) under atmosphere of N_2 containing 5 per cent CO_2 by the acid liberated as a result of degradation of penicillin G by the enzyme amidase. Simultaneously, routine method of estimation of 6-APA by iodometry was carried out as an additional check. Typical results of manometric estimation with different concentrations of the enzyme solution are given in Table I. Protein nitrogen of the enzyme solution was estimated by the modified Folin method[5] with crystalline trypsin as standard.

Table I shows that considerable amount of carbon dioxide was liberated by the blanks as compared with reaction mixture. Before dialysis, there was practically little difference in the amount of carbon dioxide evolved by the enzyme blank and the test reaction mixture after a period of 30 min. Whether this liberation of carbon dioxide is due to the persistence of ions in the dialysed solution is still to to established. Nevertheless the experiments were repeated a number of times in different concentration ranges and at different pH values. The results were in agreement with those obtained by iodometric method to an accuracy of $\pm 10\%$ (Table II). As further proof of the enzymatic splitting, unconverted penicillin G was removed with butylacetate at pH 2.5-3.0 from the solution in the Warburg flask. The aqueous phase which contained 6-APA showed antibacterial activity only when reacted with phenacetyl chloride at a pH just above neutral point.

The manometric method of estimation of amidase activity has enabled us to study some properties of the enzyme. Details will be published elsewhere.

TABLE II

AMIDASE ACTIVITY

Fraction	Activity in u/ml.	
	Iodometric	Manometric
I	1.3	1.5
II	1.4	1.3
III	0.9	1.0

Unit of amidase is defined as the amount of enzyme capable of hydrolysing 1 μm. of Na-penicillin G per hr. at 37° and pH 7.5.

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Semicontinuous Penicillin Fermentation

IN an earlier communication[1] we reported that penicillin fermentation could be prolonged for nearly 12 to 15 days by feeding a suitable mixture of sucrose and phenylacetic acid. Attempts were therefore made to see if it would be possible to withdraw 10 per cent of the mycelial suspension in broth everyday and replenish the volume with an equal volume of sucrose-precursor solution, after a substantially high penicillin titre was reached. Results of these experiments are presented below.

Strain : *Penicillium chrysogenum* HA-6, developed from Russian "New Hybrid".

Fermentation medium :

Component		*g./l.*
Peanut meal	..	30.0
Sucrose	..	3.0
$CaCO_3$	..	5.0
Cornsteep liquor	..	5.0
Phenylacetamide	..	0.5
Na_2SO_4	..	1.3
$MgSO_4.7H_2O$	..	0.06

pH was adjusted to 6.6. Fermentation details have been described in the earlier communication.[1] Sucrose-precursor mixture was fed from 24 hr. onwards, at sucrose feed rate corresponding to 0.031 per cent per hour and precursor feed rate of 0.0008 per cent per hour.

In the first experiment 1 ml. of sucrose-precursor mixture (37.5 per cent sucrose and 1.0 per cent precursor, pH adjusted to 7.0) was fed every 12 hr. up to 96 hr., when penicillin titre was 1,870 u/ml. Withdrawals of 10 ml. portions every 24 hr. were begun at this stage and the volume of the medium was replenished by adding 5 ml. of diluted (1:5) sucrose-precursor mixture solution every 12 hr.

It will be seen from Table I that in spite of withdrawals of as much as 10 per cent of the volume of the medium every day and diluting it with precursor-sucrose solution only, the penicillin production slowly increased to give a titre of 2,660 u/ml. on the 7th day. Thereafter the titre remained practically constant until the 12th day. It decreased slightly on the 13th day. Fermentation was stopped on the 14th day. At the end of the experiment 100 ml. of the fermentation beer was left having a penicillin titre of 2,240 u/ml.

TABLE I

SEMICONTINUOUS FERMENTATION OF PENICILLIN WITH SUCROSE-PRECURSOR MIXTURE FEEDING

Days of Fermentation	pH	Penicillin u./ml.	Progressive No. of 10 ml. withdrawals
4	7.1	1,870	1
5	7.1	2,270	2
6	7.2	2,510	3
7	7.2	2,660	4
8	7.2	2,560	5
9	7.2	2,640	6
10	7.1	2,660	7
11	7.0	2,560	8
12	7.0	2,510	9
13	7.0	2,270	10
14	7.0	2,240	—

Withdrawals and dilutions began on the 4th day. Sucrose feed-rate, 0.031 per cent per hour. Precursor feed-rate, 0.0008 per cent per hour.

In the above experiment a total of 100 ml. (10 x 10 ml.) with a calculated average titre of 2,450 u/ml was withdrawn in 10 days. This amounts to carrying out a fermentation for 14 days with an output equivalent to two batches of seven-day fermentations with titres of approx. 2,400 u/ml. Normally in seven-day fermentation in shaken flasks with either lactose or sucrose fed medium, the penicillin titre is about the same in 100 ml. medium. It has thus been possible to obtain, so to say, two equally efficient fermentations with only one operation for seed making and sterilization and using the raw materials for only one batch excepting for additional sucrose and precursor.

In the second experiment (Table II) withdrawals and dilutions were made beginning from the 8th day when a penicillin titre of 3,380 u/ml. was reached. It was possible to make five withdrawals (total 50 ml.) with an average titre of about 3,500 u/ml. Penicillin titre began to decline after the 13th day and the experiment was stopped on the 15th day when the titre was 2,240 u/ml. If this batch was harvested on the 13th day with a titre of 3,400 u/ml. the total production would have been equivalent to one and a half batches of fermentation with a penicillin yield of approx. 3,400 u/ml. The total units of penicillin in one and a half batches would be 150 x 3,400 = 510,000. Two such fermentations would give a total yield of 1,020,000 units. During the same period 3 normal batches of fermentation with an average titre of 2,600 u/ml. will give approx. $3 \times 100 \times 2,600 = 780,000$ units. The overall increase in production by semicontinuous fermentation over batch method would, therefore, be about 30 per cent with considerable economy in raw materials as well as operations.

TABLE II

SEMICONTINUOUS FERMENTATION OF PENICILLIN WITH SUCROSE-PRECURSOR MIXTURE FEEDING

Days of Fermentation	pH	Penicillin u./ml.	Progressive No. of 10 ml. withdrawals
8	7.1	3,380	1
9	7.1	3,540	2
10	7.1	3,640	3
11	7.1	3,510	4
12	7.0	3,400	5
13	7.0	3,400	6
14	6.9	2,850	7
15	6.8	2,240	—

Withdrawals and dilutions began on the 8th day.
Sucrose feed-rate, 0.031 per cent per hour.
Precursor feed-rate, 0.0008 per cent per hour.

If semicontinuous fermentation is successful in commercial practice, then the broth withdrawn every day could be filtered along with a regular batch that is harvested for extraction and crystallization.

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Improved Procedure for the Preparation of *N,N′-n*-Dioctylethyleuediamine

THE penicillin G salt of *N,N′-n*-dioctylethylenediamine (Octacillin*) has been found to be quite stable, and has properties desirable in an effective oral penicillin preparation. It has also been claimed to have antihistaminic properties.[1, 2] The methods reported in literature for the preparation of *N, N′-n*-dioctylethylenediamine are by

(A) the direct action of *n*-octyl chloride or ethylenediamine[3] ;

(B) condensing ethylene chloride with octylamine[4] ; and

(C) Hinsberg's procedure for the alkylation of amines.[2]

Methods (A) and (C) were repeated. The former method invariably gave an oil, while the latter procedure proved cumbersome. Considering the potential therapeutic value of Octacillin, a more facile method for the preparation of *N,N′-n*-dioctylethylenediamine in crystalline form and in better yields, was worked out.

Anhydrous ethylenediamine was prepared by distilling the commercially available variety over caustic soda,[5] and then over metallic sodium. Octyl iodide was prepared by the standard procedure.[6] It was observed that when octyl iodide was used in excess (2.2-2.3 mol.) a product of better quality and in higher yield was obtained. Different *n*-octyl halides were tried with dry ethylenediamine in various solvents. The results are presented in Table I. The procedure which gave the best results is described below.

* Trade name of Hindustan Antibiotics Ltd., Pimpri, for *N,N′-n*-dioctylethylenediamine salt of penicillin G.

TABLE I

	Reactants and their molar ratios	Solvent†	Yield of crystalline base %
1.	Ethylenediamine and octyl chloride (1:2)		<10
2.	,,	Pyridine	,,
3.	,,	Piperidine	,,
4.	,,	Ethyl alcohol	,,
5.	,,	Butyl alcohol	,,
6.	,,	Ethyl cellosolve	30-33
7.	,,	Methyl cellosolve	30-35
8.	Ethylenediamine and octyl bromide (1:2)		<10
9.	,,	Ethyl alcohol	,,
10.	,,	Pyridine	,,
11.	,,	*p*-xylene	10-15
12.	,,	Benzene	<10
13.	,,	Butyl alcohol	10-15
14.	Ethylenediamine and octyl iodide (1:2)	Butyl alcohol	35-40
15.	,,	Ethyl cellosolve	,,
16.	,,	Methyl cellosolve	30-35
17.	,, (1:2.2)	,,	40-45
18.	,, (1:2.3)	,,	60-65
19.	,, (1:2.4)	,,	42-45
20.	,, (1:2.6)	,,	30-33
21.	,, (1:2.8)	,,	28-30
22.	,, (1:3)	,,	30-32

† All solvents were dried and distilled before use.

EXPERIMENTAL

Anhydrous ethylenediamine (15.0 g., 0.25 mol.) and *n*-octyl iodide (138 g., 0.56 mol.) were added to anhydrous methyl-cellosolve (40 ml.). The solution was refluxed for 3 hr. at 130-35° with efficient stirring ; solid started separating within 10 min. The mixture was cooled and directly poured on aqueous 2*N* caustic soda solution (it can also be diluted with ether and filtered directly if the hydroiodide of the base is desired). On stirring the solution for 30 min. a colourless product was formed which was collected by filtration. The solid was washed with distilled water, dried, and crystallized from ether in colourless plates, m. p. 63-64°. (Found: N, 9. 89 ; $C_{18}H_{40}ON_2$ requires N, 9.85 per cent. Yield 46.1 g. (65 per cent). Its mixed melting point with the product prepared according to the Hinsberg's procedure showed no depression. If the product is dried at room temperature (about 26-27°) under 2 mm. pressure it gets converted to an oil. The dihydrochloride was prepared by the standard procedure ; m.p. 274-75° (dec.) (Found : N, 8.3 ; Cl, 19.9 ; neut. equiv. 177 ; $C_{18}H_{40}ON_2$ 2HCl requires N 7.8 ; Cl, 19.9 ; neut. equiv. 178.5). Its mixed melting point with the corresponding derivative prepared from the sample obtained by different method, showed no depression.

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Synthesis of *N*,*N*′-Dialkylethylenediamines

Several ethylenediamine salts of penicillin G and V such as benzathine penicillin G[1] and V[2], and hydrabamine penicillin G[3], have useful pharmacological and therapeutic properties. Other salts in the series have been reported [4, 5] from this laboratory, and some of them are being tested. A simple and economic method for the preparation of the base *N*, *N*′-dialkylethylenediamine is, therefore, of commercial interest.

One of the general methods for the synthesis of *N*, *N*′-disubstituted ethylenediamines is by the direct condensation of an organic amine with ethylene halide[6]. Ethylene dichloride being cheap and easily available, the application of the condensation for synthesis of alkyl substituted bases was investigated. *N*, *N*′-di-*n*-hexylethylenediamine dihydrochloride was thus obtained by refluxing 10.1 g. (0.1 mol.) *n*-hexylamine with 25 g. (0.25 mol.) ethylene dichloride for 3 hr. at 140-60° in an oil bath. The product (9.4 g) crystallized in the flask after 1 hr. refluxing and was recrystallized from ethanol. The recrystallized product melted at 283-85° (reported m.p. 284-86°). Analysis : Found, C, 55.3; H, 10.7 ; N, 9.3 : calculated, C. 55.8 ; H, 11.2 ; N, 9.3 per cent. The yield in the condensation was 62-65 per cent.

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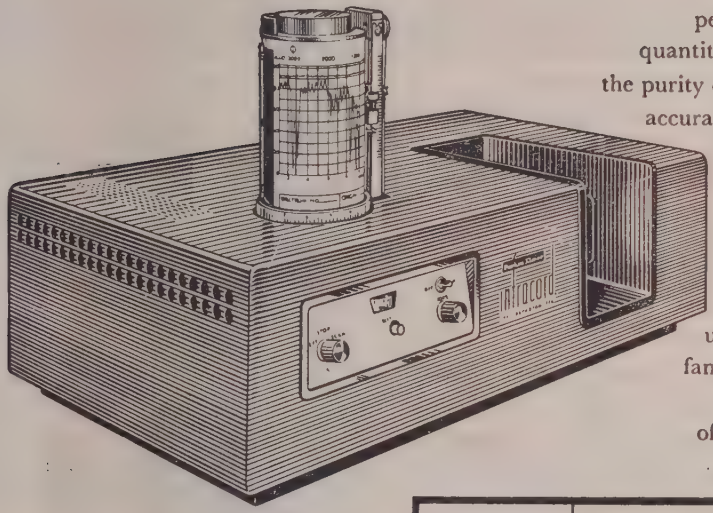

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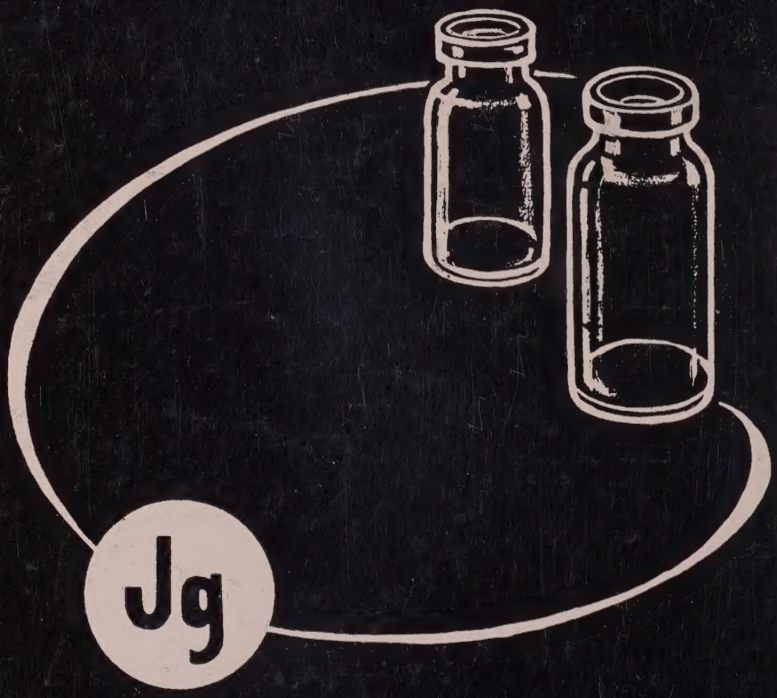

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